Neuroglobin Genetic Polymorphisms and Their Relationship to Functional Outcomes Following Traumatic Brain Injury

by

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BACKGROUND: Neuroglobin (Ngb) is a protein that increases oxygen availability in ischemic neuronal tissues, but whether Ngb gene variants contribute to patient outcomes is unknown.

PURPOSE: To identify functional or non-functional variants in the Ngb gene in severe traumatic brain injury (TBI) patients and determine whether variants impact patients' injury severity and functional outcomes. **Specific Aims:** To identify Ngb variants (present/absent) in DNA extracted from the cerebral spinal fluid and blood of patients with severe TBI, and then: 1) determine the variant frequencies, 2) determine demographic and clinical patient characteristics based on Ngb variants, 3) determine the relationship between the variants and TBI severity as measured by admission Glasgow Coma Scale (GCS), and 4) determine differences in functional outcomes (Glasgow Outcome Scale [GOS]) at 3,6,12, and 24 months post TBI based on Ngb variants.

METHODS: Prospective, descriptive, comparative design using DNA collected (NIH NR04801 and NS30318) from 196 Caucasian subjects (non-Caucasians excluded to eliminate confounding from ancestry). We generated Ngb genotype data for 2 tagging single nucleotide polymorphism (SNP) variants (captures all of Ngb's genetic variation) using TaqMan PCR technology. Data analysis: independent t-tests; Fisher Exact, Pearson's Chi-square, Exact tests; logistic and linear regression.

RESULTS: For Ngb SNP1, 36.3% were CC/CT (non-wild typed or present variant [SNP1 Vpresent]), and 62.2% were TT (wild typed or absent variant [SNP1 Vabsent]). For Ngb SNP2, only 6.6% were TT/GT (SNP2 Vpresent), whereas 91.3% were GG (SNP2 Vabsent). There was no significant relationship between variants of SNP1 or SNP2 and either demographic or clinical characteristics. There was a trend toward significance between SNP1 Vabsent and better GCS ($p = 0.061$), but not between SNP2 variants and GCS ($p = 0.109$). Subjects with good outcome by GOS were more likely to be SNP1 Vabsent at 3, 6, 12, and 24 months ($p = 0.023$; $p = 0.01$; $p = 0.002$; $p = 0.035$ respectively). No significant relationship was found between SNP2 and GOS at any time point. Using logistic and linear regression controlling for age, gender, and GCS, SNP1 Vpresent was significantly associated with poorer GOS at 12 months ($p = 0.028$) only; SNP2 showed no significance in regression analysis.

CONCLUSION: SNP1 Vabsent TBI patients were more likely to have good outcomes, whereas genetic variants of SNP2 did not impact outcomes; possibly because Ngb SNP1 Vabsent affects the quantity or quality of Ngb in severe TBI, producing better outcomes.

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PREFACE

I am grateful to all of the TBI patients and their families who contributed their support and encouraged this research at the Neuro-Trauma Intensive Care Units of UPMC. I also thank Dr. Marry Kerr for introducing me to my topic and for guiding me in my research. Dr. Kerr motivated me to challenge myself as I studied the fields of pathology, protein structure, and the genetics of neuroscience.

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1.0 INTRODUCTION, PURPOSE, AND CONCEPTUAL FRAMWORK

1.1 INTRODUCTION

About 1.5 million people sustain traumatic brain injury (TBI) in the U.S. each year, at the rate of approximately one person every 20 seconds. Of the patients who sustain TBI annually, 50,000 die, 235,000 are hospitalized, and 1.1 million are treated and released in emergency departments (EDs) (Langlois, J. A., Rutland-Brown, W., Thomas, K. E., 2006). Locally, there are 245,621 people living with TBI injuries in Pennsylvania; yearly, about 2,223 die, 10,463 are hospitalized, 49,505 are seen in the Emergency Room, and about 8,612 sustain long term or life-long disabilities due to brain injury (2007). These data show the importance of TBI as a major cause of morbidity and mortality both nationally and locally. Certain sociodemographic characteristics have been identified as risk factors for TBI. The leading causes of TBI are falls (28%), motor vehicle crashes (20%), striking/being struck by something (19%), assaults (11%), others (7%), suicide (1%), other transport (2%), bicycle or non motor vehicle (3%), and unknown (9%), respectively (Langlois, J. A., Rutland-Brown, W., & Thomas, K. E., 2004). TBI occurs more commonly in males (twice frequently than in females) (Coronado, V. G., Thomas, K. E., & Kegler, S. R., Morb Mortal Wkly Rep, 2007). Furthermore, adults aged 75 years or older have the highest rates of TBI-related hospitalization and death (Langlois, Rutland-Brown & Thomas, 2004). Regarding ethnicity, African Americans have the highest rates of death and

hospitalization from TBI (Langlois, Rutland-Brown, & Thomas, 2006). Therefore, while gender affects TBI prevalence, age and race affects the probability of mortality after TBI.

Traumatic brain injury can have a significant financial and physical consequence on its victims. Direct medical costs and indirect costs such as lost productivity due to TBI totaled an estimated $60 billion in the United States in 2000 (Finkelstein, Corso, Miller, et al., 2006). TBI hospitalization rates have increased from 79 per 100,000 in 2002 to 87.9 per 100,000 in 2003 (Coronado, V. G., Thomas, K. E., & Kegler, S. R., Mobility and Mortality Weekly Report, 2007). These costs are likely due to the wide range of functional impairments due to TBI that affect thinking, sensation, language, and/or emotions. Forty percent of patients hospitalized with a TBI report problems with memory, decision making, stress management, emotional stability, and job skills. At least 5.3 million Americans, 2% of the US population, currently live with disabilities resulting from TBI. Furthermore, moderate and severe head injuries are associated with a 2.3 and 4.5 times increased risk of Alzheimer's disease, respectively (Langlois, Rutland-Brown, & Wald, 2006).

The National Institute of Neurological Disorder and Stroke (2007) has identified the symptoms of moderate or severe TBI, which include headaches that get worse or do not go away, repeated vomiting, nausea, convulsions or seizures, inability to awaken from sleep, dilation of one or both pupils of the eyes, slurred speech, weakness or numbness in the extremities, loss of coordination, and increased confusion, restlessness, or agitation. Morbidity and mortality following TBI result from both primary and secondary injury. Primary brain injury results from direct trauma at the time of injury. Secondary injury, which can occur hours to days after a TBI, results from a wide range of later-occurring bimolecular and pathophysiological derangements within any or all of the three intracranial compartments: 1) brain; 2) cerebrospinal fluid (CSF);

and 3) blood flow (Tolias, Sgoursos, Dulebohn, Sheridan, Engelhard, & Dogali, 2005). These physiologic derangements can then cause delayed cerebral ischemia, cellular necrosis, apoptosis, and death (Tisdall, & Smith, 2007). Health care providers cannot prevent primary injury, but by preventing secondary injury, TBI patients are offered their best opportunity for recovery. However, even with such care, functional recovery is frequently less than optimal.

More recently, interventions are being explored which enhance the patients' own neuroprotective mechanisms to prevent or limit secondary injury. Ngb is emerging as one such potential neuronal protector. Ngb is a neuronal protective protein expressed in response to cerebral hypoxia or ischemia in order to assist in oxygen transport/utilization or exchange with biotoxic agents, particularly in situations where brain oxygenation is impaired. The first research project examining Ngb, a monomer with a high oxygen affinity (half saturation pressure, or P50, of approximately 2 torr), was reported by Burmester Weich, Reimhards, and Hanker in 2000. Burmester and his colleagues reported that Ngb exists in the central and peripheral neural systems, as well as the endocrine systems and retina, the areas with the highest oxygen energy needs in humans. Since 2000, some animal, but few human studies have been conducted to explore the role that Ngb may play as a protective or compensatory mechanism during cerebral ischemia. Sun and Jin (2003) suggested that *in vivo*, Ngb protects the brain from experimentally induced strokes by reporting a positive correlation between the less infarct volume with more Ngb mRNA expression or increased Ngb protein level in mice and rat models.

Whether or not Ngb is present, or has a beneficial function in humans during brain ischemia has not yet been determined. However, a gene for Ngb has been identified in humans. An initial first step in examining the role that Ngb may play in protecting the human brain against ischemia would be to investigate and identify genetic variants or polymorphism functions

of the Ngb gene in patients who have suffered from a TBI. This would determine whether or not genetic variations in this gene contribute to clinical outcomes. If such genetic variants were to be noted, then further work to identify the protein itself would be warranted.

1.2 PURPOSE OF THIS STUDY

The purpose of this study is to investigate and identify functional and non-functional variants in the Ngb gene in severe TBI patients and to determine whether these variants play a role in either the patients' severity of injury or clinical outcomes.

1.3 SPECIFIC AIMS

The specific aims of this study are to:

Specific Aim 1. Determine the frequency of Ngb variants in DNA extracted from the CSF and blood of patients with severe TBI.

Specific Aim 2. Determine demographic and clinical characteristics of patients based on the presence or absence of Ngb variants in the TBI population.

Specific Aim 3. Determine the relationship between Ngb variants (present/absent) and the severity of TBI as measured by the admission Glasgow Coma Scale (GCS).

Specific Aim 4. Determine potential differences in the functional outcomes (Glasgow Outcome Scale (GOS) of patients with and without the Ngb variants (good outcome = GOS 4-5; poor outcome = GOS 1-3) at 3, 6, 12 and 24 months post injury.

1.4 DEFINITION OF TERMS CONCEPTURAL FRAMEWORK

***Neuroglobin single nucleotide polymorphism (SNP)*:** is a DNA sequence variation of Ngb involving a single nucleotide. Almost all common SNPs have only two alleles.

***Traumatic Brain Injury (TBI)*:** Sudden insult to the brain by an external physical force which alters the state of the skull, brain tissue, and brain blood flow resulting in a change in the level of consciousness and the impairment of cognitive, psychosocial, and emotional functions.

Glasgow Coma Scale (GCS): A neurological scale that assesses the conscious state of a person following head injury with eye (E4), verbal (V5), and motor (M6) responses. The GCS ranges from a total possible GCS score of 15 to a minimum score of 3. The three accepted classifications are: severe TBI (GCS < 8), moderate TBI (GCS 9-12), and minor TBI (GCS > 13) (Teasdale G, Jennett B, 1974). One of the entry criteria for this study was a severe TBI with a score of ≤ 8.

Severity of TBI: Further degrees of TBI severity for study subjects was indicated by a score along the continuum of severe TBI 3-8 on the Glasgow Coma Scale as a categorical variable, or as a dichotomous variable (GCS better = score 6-8; GCS poor = score 3-5)

Glasgow Outcome Scale (GOS): Measurement of a patient's functional outcome. The GOS was developed by Drs. Jannett and Bond in 1975 with five categories: 1 = dead, 2 = vegetative, 3 = severely disabled, 4 = moderately disabled and 5 = good recovery. Scores were also designated as good (GOS 4-5) and poor outcome (GOS 1-3).

1.5 CONCEPTURAL FRAMEWORK

The degree of primary injury from TBI is dependent upon the mechanism of injury and demographic patient characteristics. Once sustained, the level of TBI is characterized as mild, moderate or severe based upon the GCS, with a GCS score of 3-8 denoting severe injury. Thereafter, secondary injury due to cerebral hypoxia or ischemia ensues due to impaired brain oxygen delivery, which results in the disruption of brain cellular metabolism, thereby leading to brain cellular impairment or death. In the clinical setting, Ngb is hypothesized to assist with the transport of oxygen to brain cells, or with protection from cellular toxins. Improvement of brain oxygen delivery and protection against injury should result in better cellular function and, in turn, better functional outcomes. As shown in Figure 1, this study investigated if variants in the Ngb gene (present/absent) plays a role in the TBI patients' severity of injury as measured by GCS, as well as functional outcomes (good functional outcome = GOS 4-5; poor functional outcome = GOS 1-3) in the 3, 6, 12, and 24 months following TBI. Based upon a review of the literature, we anticipate that patients with genetic variants in Ngb SNP1 and SNP2 will have different outcomes at 3, 6, 12, and 24 months following TBI.

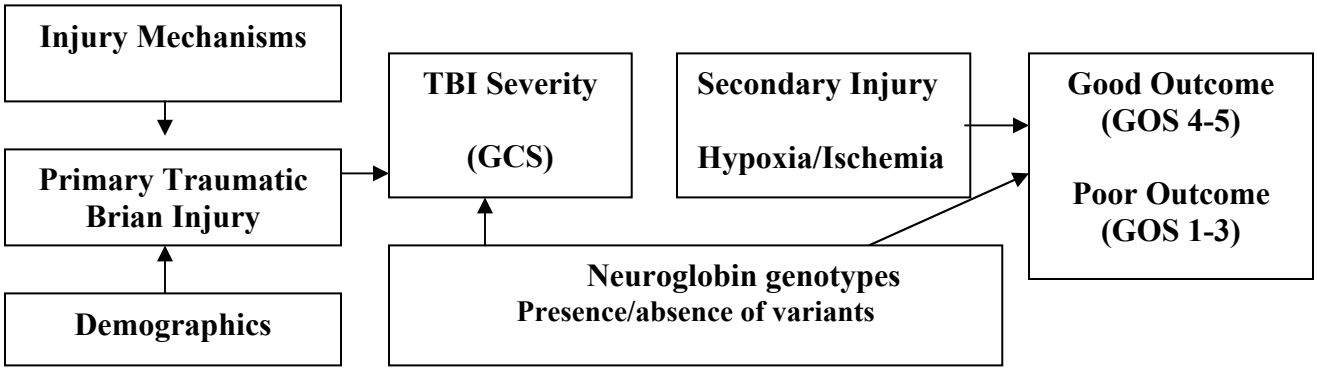


Figure 1. Conceptual Model

2.0 BACKGROUN AND SIGNIFICANCE

Chapter two describes the state of knowledge regarding the TBI and neuroglobin literature to date supporting the theoretical framework of this study. Part one, which interprets severe TBI, focuses on: 1) pathologic changes in the brain, CSF, and CBF promoting secondary injury; 2) mechanisms in cerebral hypoxia and ischemia; and 3) the comparison of mechanisms of TBI and stroke. Part two focuses on 1) the structure of Ngb and globins; 2) Ngb's mechanisms during hypoxia and ischemia; 3) genetic findings for Ngb; and 4) published studies dealing with the relationships between secondary injury (hypoxia/ischemia) and Ngb in TBI.

2.1 BRAIN HEALTH MONITORING IN TRAUMATIC BRAIN INJURY

Garry (2003) stated that "the adult human brain contains more than 100 billion neurons with over 10^{15} synapses, and requires more than 10-15% of the cardiac output to maintain function and consciousness" (Mammen, 2003; Slavik, & Rhoney, 2000, p.342). Normally, brain tissue, which makes up only 2% of body weight, consumes about 20% of the available oxygen during rest (Burmester, Weich, et al., 2000; Moens, L. & Dewilde, S., 2000, Zhang, C., Wang, C., et al., 2002). The three main components of the brain are brain tissue (80-90%), cerebrospinal fluid (CSF; 3-10%), and cerebral blood flow (CBF; 10%) (Slavik, R. S., & Rhoney, D. H., 2000). The

basic brain structures includes cerebrum, brain stem (midbrain, pons, and medulla), and cerebellum. The brain is bathed in cerebrospinal fluid (CSF), which circulates between layers of the meningitis and through the brain ventricles. Cerebral blood circulation occurs primarily through the Circle of Willis, (including anterior cerebral arteries, internal carotid artery, posterior cerebral arteries, posterior communicating arteries (left and right) and anterior communicating artery.

2.1.1 Monitoring mechanisms

According to the National Institute of Health (NIH) and its sponsored Traumatic Coma Data Bank (TCDB), TBI is considered severe when the GCS drops below 9 within 48 hours of injury and when CT scans reveal abnormalities (Dawodu, S.T., 2007). The alterations in the three compartments (brain, CSF, and CBF) appear as major pathologies in those patients with severe TBI.

Patients must be monitored for the development of brain herniation, which is the displacement of brain tissue, cerebrospinal fluid, and blood vessels outside the compartments they normally occupy (Crippen, D. W., & Shepard, S., 2008). A herniation can occur at the base of the skull through the foramen occipital, or through openings created by a craniotomy procedure. Herniation is considered a medical emergency requiring immediate treatment to prevent death.

TBI leads to increased mortality when the intracranial pressure (normal ICP = 0-15 mmHg) increases. When the ICP increases, signs and symptoms of neurological impairment are present, and immediate interventions are necessary, including CSF drainage and neurological surgery. In order to assess the progression of brain damage, a CT examination is frequently

necessary. Marshall, Marchall, & Klauber (1991) developed four categories of brain injury severity based upon CT examination results: 1) Diffuse Injury 1 = lack of evidence for any significant brain injury; 2) Diffuse Injury 2 = either identifies a midline shift of less than 5 mm or widely present CSF cisterns at the base of the brain; 3) Diffuse Injury 3 = a midline shift of less than 5mm with partial compression or absence of the basal cisterns and no high or mixed-density lesion volumes greater than 25 ml; and 4) Diffuse Injury 4 = a midline shift greater than 5mm, a compression and absence of the basal cistern, and no lesions of high or mixed density greater than 25 ml. CSF is manufactured by the brain at a rate of approximately 500 ml per day and circulates within the internal brain compartments and around the spinal cord. Analysis of the CSF provides important information about the metabolic functions of the brain, and aids in the diagnosis of brain hypoxia or ischemia. The CSF pressure, ranging from 6 -10 mmH_20 (4.4-7.3 mmHg), contains only 0.3% (25 mg/dl) plasma protein (Felgenhauer, K., 1974, & Saunders N.R., Habgood, & Dziegielewska, K.M., 1999).

Fifteen percent of cardiac output is distributed to the brain; with normal cerebral blood flow (CBF) defined as approximately 750 ml/min. The CBF, equivalent to 40-50 ml/100g/min, can be calculated by dividing cerebral perfusion pressure (CPP) by cerebrovascular resistance (CVR). CPP is determined by the mean arterial pressure (MAP) and intracranial pressure (ICP). Therefore, CPP is equivalent to MAP (normally 50-150 mmHg) minus ICP (normally less than 15 mmHg) (Slavik & Rhoney, 2000). A normal CVR (derived by subtracting the diastolic pressure from the systolic pressure and then dividing by the MAP) is regulated by metabolic control, pressure auto regulation, chemical control (partial pressure of oxygen (PaO_2) and partial pressure of carbon dioxide ($PaCO_2$), and by neural control. When the CBF falls to 18-20 ml/100g/min, cerebral ischemia becomes irreversible. Brain tissue death occurs when CBF falls

under 8-10ml/100g/min. When the systemic MAP falls below 50 mmHg, cerebral ischemia due to low CPP may ensue. In contrast, if the MAP becomes higher than 150mmHg, the brain can experience capillary injury or cerebral edema.

CPP=MAP-ICP ***CBF= CPP/CVR*** ***CVR = SBP-DBP/MAP***

The metabolic rate of the brain is often expressed in terms of its rate of oxygen consumption ($CMRO_2$), which is normally 3.5 ml/100g brain/min. The extraction of oxygen from cerebral blood ($CEO_2 = SaO_2\text{-}SjvO_2$; normally 24-42%) is defined as the change between arterial oxygen saturation (SaO_2; normally = 100%) and jugular venous oxygen saturation ($SjvO_2$; normally = 55-71%). The cerebral extraction ratio of oxygen ($CERO_2 = CMRO_2/CDO_2$; normally = $35 \pm 10\%$) is calculated by dividing oxygen consumption (or $CMRO_2$) by the cerebral oxygen delivery (CDO_2) to equal $(SaO_2\text{-}SjvO_2)/SaO_2$. Thus, both brain oxygen consumption as well as brain oxygen delivery influences the $CERO_2$ (Slavik & Rhoney, 2000).

In addition, arterial blood gases (ABGs) are used to assess both cerebral and systemic partial pressure of gases and other substances that are being delivered to the brain. Components measured are the range of the partial pressure of arterial oxygen (PaO_2 = 80-100 mmHg) and carbon dioxide ($PaCO_2$= 35-45 mmHg), bicarbonate concentration (HCO_3^- = 22-26 mmol/l), the blood bicarbonate concentration at a CO_2 of 5.33 kPa, with full oxygen saturation at 37°C (standard bicarbonate concentration; SBCe= 21 to 27 mmol/l), the base excess (BE = -2 to +2 mmol/l); phosphate concentration (HPO_4 = 0.8 to 1.5 mM) (Walter, 2001), and blood pH (7.35-7.45). The ABG is an additional tool which helps clinicians to observe the adequacy of CBF variation in the acute, sub acute, and reperfusion phases.

In summary, the brain is an organ whose health is dependent upon complex interactions between the brain tissue, CSF, and CBF. Furthermore, during cerebral hypoxia or ischemia when

a deficiency of oxygen, glucose, and blood flow occurs, the brain can move very rapidly from aerobic to anaerobic metabolism (Slavik & Rhoney, 2000).

2.2 HYPOXIA/ISCHEMIA PATHOLOGY IN TBI

Cerebral hypoxia is the deprivation of normal oxygen supply to brain tissue, while cerebral ischemia occurs when brain tissue does not receive enough blood flow in order to maintain normal neurological cellular function. Both cerebral hypoxia and ischemia bring about poor outcomes in patients with severe TBI. The lack of brain oxygen forces anaerobic glycolysis, which begins a cascade of events. The cerebral cortex (cerebral gray matter) and the hippocampus (forebrain and temporal areas) are the most vulnerable regions in the brain in terms of ischemia injury (Shang, Zhou, Wang, Gao, Fan, & Wang, et al., 2006) and are the primary areas of interest in studies of brain hypoxia and ischemia involving animal models. Numerous cellular and molecular mechanisms are induced in response to a hypoxic or ischemic insult.

2.2.1 Causes of cerebral hypoxia/ischemia in TBI

Within the ischemic cerebrovascular bed, there are two major zones of injury: the core ischemic zone and the "ischemic penumbra" (the term generally used to define ischemic, but still viable, cerebral tissue) (Lipton, 1999). Measuring CBF is a simple and physiologically meaningful way to differentiate between the regions of the cerebral core and penumbra in the brain. In the core zone, an area of severe ischemia, blood flow is below 12ml/100g/min, and the loss of inadequate supply of oxygen and glucose results in a rapid depletion of energy stores. Severe ischemia can

result in necrosis of neurons as well as supporting cellular elements (i.e. glial cells) within the severely ischemic area. Brain cells within the penumbra, a rim of mild to moderately ischemic tissue lying between tissue that is normally perfused and the area, in which the infarction is evolving, may remain viable for several hours. That is because the penumbral zone is supplied with blood by collateral arteries anastomosing with branches of the occluded vascular tree. However, cells in this region will die if reperfusion is not established during the early hours since collateral circulation is inadequate to keep up with the neuronal demand for oxygen and glucose indefinitely. The penumbral area is associated with cerebral hypoperfusion in the range of with 15-18 ml/100g/min. Hypoperfusion can result in the malfunction of the Na^+/K^+ ion pump, which exchanges ions between the inside and outside of cells at a CBF of 10-15 ml/100g/min, with cellular calcium influx initiated at a CBF of 6-15ml/10g/min (Leker & Shohami, 2002). While the cerebral core is comprised of cells that have progressed to necrosis and are in an irreversible state, the penumbra is in a reversible state. Both of these states develop within one to 24 hours.

CBF can also be used monitor the progression of cerebral edemas (cytotoxic or cellular edema and vasogenic edema) (Leker, & Shohami, 2002; Hassmann, 2006). Cytotoxic edema evolves over minutes to hours and may be reversible, while the vasogenic phase occurs over hours to days. Cytotoxic edema is characterized by the swelling of cellular elements of the brain. In the presence of acute cerebral ischemia, neurons, glia (indicated by astrocytes), and endothelial cells swell within minutes of hypoxia due to failure of ATP-dependent ion (sodium and calcium) transport. With the rapid accumulation of sodium within cells, water follows so as to maintain osmotic equilibrium. The resulting increased intracellular calcium activates phospholipases and the release of arachidonic acid, leading to the release of oxygen-derived free radicals and infarction (Fishman, 1992). Vasogenic edema is caused by an increase in

extracellular fluid volume due to the increased permeability of brain capillary endothelial cells to serum proteins, especially albumin. Acute hypoxia initially causes cytotoxic edema, followed within the next hours to days by the development of vasogenic edema as the infarction develops (Fishman, 1992). The delayed onset of vasogenic edema suggests that time is needed for the defects in endothelial cell function and permeability to develop. After four to six hours of hypoxia or ischemia, tissue necrosis results causing proteins to break down and pass through the blood-brain barrier (BBB).

Both diffusion-weighted magnetic resonance imaging (MRI) (DWMRI) and perfusion-weighted MRI (PWMRI) can also be utilized to distinguish primary injury in the acute phase (Leker, & Shohami, 2002). The DWMRI sensitively detects irreversible tissue damage (for instance, core and penumbra) and the PWMRI reflects the perfusion impairment in the region of the hypoxia or ischemia. The lower the CPP, the higher the ICP becomes due to swelling and edema of ischemic tissue.

A sensitive indicator of CBF is brain cell metabolic function. Glucose metabolism is inhibited when CBF falls below 50% of normal (about 55 ml/100g/min) and is later completely suppressed when CBF falls below 35 ml/100g/min during anaerobic glycolysis. As CBF falls below 25 ml/100g/min, tissue acidosis begins. Simultaneously, lipids rapidly break down to fatty acids, which can be toxic to cells. Acidosis is the accumulation of pyruvate and lactate, which leads to depolarization via inactivation of the Na^{+}-K^{+} pump (Hossmann, 2006). Moreover, carbon dioxide and glutamate levels are increased and the pH decreases. There is some indication that systemic hyperglycemia, defined as a serum glucose level of greater than 11.1 mmol/L, is associated with a significantly worse outcome in severe TBI patients (Slavik &

Rhoney, 2000). Whether hyperglycemia produces direct damage or is a marker of injury severity is not currently well described.

In cerebral hypoxia or ischemia, both the N-methyl-D-aspartic acid (NMDA) glutamate receptor and the α-amino-3-hydroxy-5-methylisoxazole-4- propionic acid (AMPA, non-NMDA) release glutamate into the extraplasmic space of the central nervous system (CNS). This action promotes cellular depolarization which results in an influx of sodium (Na^+), chloride (Cl^-), and calcium (Ca^{++}) in exchange for potassium (K^+) and hydrogen carbonate (HCO_3^-) (Johnston, Trescher, Akira, & Wako, 2001; Slavik, & Rhoney, 2000; Leker, Shohami, 2002; Hassmann, 2006; Drake& Iadecola, 2006). Soon, depolarization activates nitric oxide syntheses (NOS) and reactive oxygen species (ROS).

The three kinds of isoformed NOS are neuronal (nNOS), inducible (iNOS), and endothelin (eNOS). NOS produce nitric oxide (NO) arginine in the central nervous system (CNS) (Leker, & Shohami, 2002). Both NMDA and AMPA enhance ROS and NOS by increasing cellular calcium influx (Johnston, Trescher, Ishida, & Nakajima, 2001). The ROS produces both hydrogen peroxide (H_2O_2) through the superoxide dismutase (SOD) and the superoxide (O_2^-) from cytosol during cellular depolarization by increasing calcium influx into the cells. The SOD catalyzes O_2^- into oxygen (O_2) and H_2O_2. Other NOSs (iNOS, eNOS, and nNOS) produce intracellular NO, including in the mitochondria. NO immediately combines with O_2^- to become peroxynitrite ($ONOO^-$). $ONOO^-$ has a high affinity for CO_2. The two combine to become nitrosoperoxocarboxylate ($ONOOCO_2^-$), the worst toxic gas. Toxic gas, such as H_2O_2, O_2^-, NO, $ONOO^-$, or CO_2, causes DNA fragmentation and death, which in turn causes apoptosis or necrosis. Normally, NOS do not cause any damage in aerobic cells but will harm anaerobic cells.

When the mitochondrial matrix swells as a result of increased NOS and ROS, it releases the pro-apoptosis proteases cytochrome c, caspase 3/7, and calpain. Also, a higher level of calcium induces damage through the mitochondrial permeability transition (MPT) and also damages DNA fragments. Later on, cell death occurs, induced by either apoptosis or necrosis. Caspases 3 and 7 cause the maturation of cytokines and are found active in the first 5 hours (~11 fold) and 24 hours (16 fold) after hypoxia development in neuronal cells (Rayner, Duong, Myers, & Witting, 2006). The mitochondrial membrane is one of the largest components of the incomplete O_2 respiration reduction. Additionally, lissome and astroglia are also involved in anaerobic injury (Rayner, Duong, Myers, & Witting, 2006).

2.2.2 Neuroprotector functions

"Good" biochemical and molecular mechanisms in hypoxia and ischemia include the poly (ADP-ribose) polymerase (PARP), a nuclear enzyme that is strongly activated by repair DNA. Secondly, cystatins, cysteine proteinase inhibitors (such as cathepsin B, H, or L), are intracellular lysosome and microglia within the brain that provide protection from hypoxia and ischemia (Wakasugi, Nakano, et al., 2004). For example, cyctatin b protects against neuronal death under oxidative stress or other chronic neurodegenerative diseases (Alzheimer's disease; AD). Surface Plasmon Resonance (SPR) is a powerful tool for real time measurements of direct protein-protein interactions in the study of mice. Furthermore, flotillin-1 is a lipid raft microdomain, expressed in neurons and astroglia associated with the plasma membrane and distinct intracellular components of Na^+-K^+ATPase and protein β_2 subunits (Wakasugi, K., Nakano, T. et al., 2003, Hankeln, T., Wystub, S. et al., 2004, Wakasugi, K., T. Nakano, et al., 2004). Spectrin and ankyrin are major cytoskeletal proteins that provide a link between cell membrane and

membrane-associated proteins. Ankyrin plays a linking the Na^+-K^+ATPase and Na^+ channels to the cytoskeleton.

Furthermore, erythropoietin (EPO), a kidney-derived glycoprotein hormone that is a mitogen for erythrocyte (red blood cell) precursors in the bone marrow, also responds to oxidative stress in neurons and astrocytes under hypoxia/ischemia (Leker, & Shohami, 2002). The EPO receptor is normally present on neurons and brain capillary endothelial cells. Also, estrogen and progesterone reduce excitotoxicity and change glutamate receptor activity by decreasing immune mediated inflammation and axonal remyelinization by enhancing synaptogenesis and dendrite arborization (Leker & Shohami, 2002).

Endogenous cellular anti-oxidant defense mechanisms (detoxifying enzymes) include 1) SOD; 2) catalase; 3) low-molecular weight anti-oxidant glutathione; 4) α-tocopherol (the most potent biological form of Vitamin E); and 5) ascorbic acid (Vitamin C) (Rayner, Duong, Myers, & Witting, 2006).

Additional biochemical materials which are protective during hypoxia include hypoxia-inducible factor 1 (HIF-1), NO, cobalt chloride ($CoCl_2$), deferixiamine (Dfx) and sodium nitroprusside (SNP). Because they have a regulatory role in localized tissue, they serve as a critical pathway for tumor vascularization, myocardial ischemia, and stroke (Zhu, Y., Sun, Y., Jin, K., & Greenberg, D. A., 2002). Cathepsin, a protenase found in lysosomes, participates in neuronal degeneration resulting from ischemic insult. Oxidative stress rapidly initiates the translocation of cathepsins B and L from the lysosome to the cytosol. Ngb has been found to facilitate this transport (Wakasugi, K., T. Nakano, et al., 2004). Therefore, to diminish the cathepsin, cystatin C and flotillin directly interact within the lysosomes. TNF-α is found both in the circulation and the brain. NFkB activates the synthesis of many proteins, such as iNOS and

COX-2. Protein synthesis is extremely sensitive to cell energy charge and ion contents, including free radicals in endothelial cells. Protein kinases (PK) and phosphatases alter cell membrane, mitochondrial function, the cytoskeleton, and protein synthesis. The accumulation of neutrophils (endogenous neuroprotectants) in vessels requires interactions with several adhesion molecules, such as intracellular adhesion molecule (ICAM-1) as well as E and P-selectins on the endothelial cells, fibronectin and lamininin within the extracellular matrix, and integrins and L-selectin on the white blood cells (Lipton, 1999). Leukocytes are also activated, and can cause a reaction (free radicals produced by nicotinamide adenine dinucleotide phosphate-oxidize; NADPH) which has direct toxic effects on the vasculature or the neurons (Lipton, 1999). A positive correlation exists among leukocyte activity, the size of the infarct, and the severity of neurological outcome. Copper and zinc are essential secondary messengers which excitatory synapses release during neuronal activation (Choi & Koh, 1998) that cause histopathological damage.

Conversely, the "bad" biochemical and molecular mechanisms in hypoxia or ischemia include the Heat Shock Proteins (HSPs), which are thought to bind to denaturized proteins. In addition, inflammatory cytokines (such as TNF-α and IL-1β) appear as early as one hour after cerebral ischemia or brain trauma and act as chemo attractants to leukocytes (Ashwal, Tone, Tian, Cole, & Pearce, 1998; Barone & Feuerstein, 1999; Fan, Young, Barone, Feuerstein, Smith, & McIntosh, 1996). The NF-kappa B response to the oxidative stress in penumbra is similar to that of the protectors, while the prostaglandin synthesizing enzyme cyclo-oxygenase-2 (COX-2) occurs in neutrophils, vascular cells, and neurons (Drake & Iadecola, 2006). Furthermore, ICAM, ELAM, as well as tissue metal proteases, penetrate the leukocytes through the blood brain barrier (BBB) (Hassmann, 2006). Growth factors (GFs- nerve growth factor, brain derived

neurotrophic factor (BDNF), glial derived growth factors (GDNF), basic and acidic fibroblastic growth factors (FGF), and the transforming growth factor super family (TGF) (Leker & Shohami, 2002) all increase the magnitude of the protective effect of magnesium and hypothermia treatment. Another "bad" biochemical is calpain, which activates autophagocytosis. Autophagocytotic cell death produces lysosomes with mono- or multilayered lipid membrane vesicles that enclose regions of cytoplasm and organelles. The characteristic morphology of autophagocytotic cell death includes condensed cytoplasm, large vacuoles, lysosomes, and a nucleus with irregularly shaped chromatin. Cell death is defined as eosinophilic, shrunken neurons or neurons showing autophagocytotic morphology (Lipton, 1999).

2.2.3 Cellular death: apoptosis vs. necrosis

Cell death occurs at the point at which cells are unable to recover their normal morphology and function (Lipton, 1999). Apoptosis, a normal developmental process, differs from necrosis (Figure 2). Apoptosis is described as the orchestrated collapse of a cell, staged membrane bleeding, cell shrinkage, nuclear fragmentation, chromatin condensation, and the budding off of intact cell fragments (apoptotic bodies). Necrosis is a degenerative phenomenon produced by major environmental changes such as severe ischemia, extremes of temperature, and mechanical trauma. The characteristics of necrosis are swelling of organelles, cytoplasm, membrane disruption, and disintegration of the cell body (Rollins, Perkins, Mandybir, & Zhang, 2002).

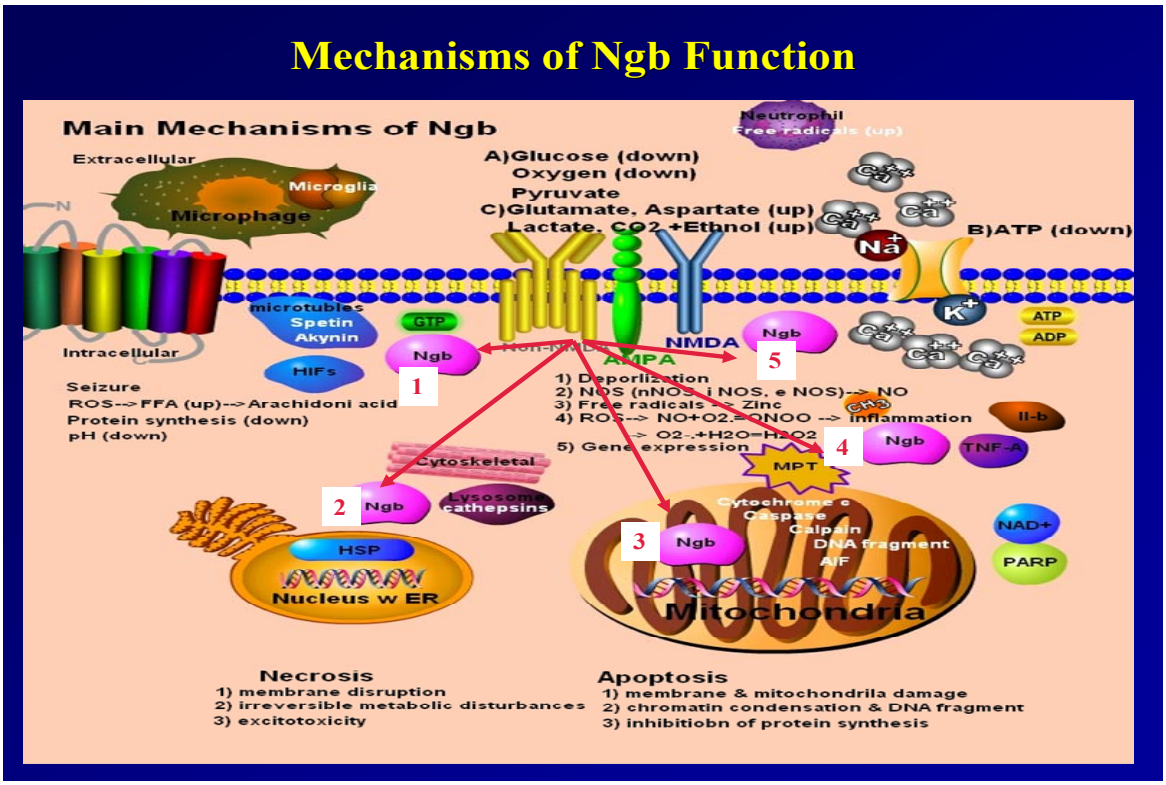


Figure 2. Illustration of the Hypoxic and Ischemic Pathway

In summary, cerebral hypoxia/ischemia results from impairment of CBF and the resulting diminished delivery of oxygen and glucose to brain cells. These events produce primary injury, but are also responsible for secondary brain injury after TBI. By understanding these events, we can identify how to limit secondary injury by searching for an endogenous neuroprotector that help improve the clinical outcomes of patients following TBI.

2.3 NEUROGLOBIN (NGB)

2.3.1 Ngb and globins

The globins have five family members: hemoglobin (Hb), Myoglobin (Mb), cytoglobin (cyto), hemoglobin X, and Ngb; all are found in bacteria, protists, plants, fungi, and both vertebral and non-vertebral animals (Burmester, Weich, et al., 2000; Sun, Jin, et al., 2001; Pesce, Dewilde, et al., 2003; Nienhaus, Kriegl, et al., 2004). Globins maintain binding, storing, transporting, scavenging, detoxification, and gas-sending functions (Dewilde & Moens, 2000; Sun, Jin, et al., 2003). Hb and Mb are normally found within the mM range, but the Ngb and Cyto are only found within the μM range (Fago, Hundahl, et al., 2004). Additionally, there is also globin X, the newest globin member, but little is known about it beyond the fact that it exists in lower vertebrates and invertebrate species.

Normally, globins have the same redox statement of octahedral coordination geometry with six potential binding sites. Heme pyrrole nitrogens occupy four equal binding sites and leave one histidine chain, which allows proximal (His96-F8: the eight amino acid along the F helix with the globin structure; fifth coordination) and distal (His64E7; sixth coordination) coordination with the central iron atom (ferrous, Fe^{2+}; ferric, Fe^{3+}) (Trent III, Watts, & Hargrove, 2001). In general, globins are observed in a pentacoordinated (deoxygenated) state except when some pathological situation, such as acute or chronic hypoxia or ischemia, induces a change to the hexacoordinated (oxygenated) state (Pesce, Bolognesi, et al., 2002). Therefore, when the distal coordinate is empty without any endogenous or exogenous (external) ligands, it is in the pentacoordinate state. In contrast, the six-sided hexacoordinate state has already bonded with endogenous or exogenous ligands. The oxygen binding and dissociation rate balance the

proximal (His96-F8) and distal (His64-E7) with the heme (iron). The ligand binding competition is equal, although His96-F8 and His64-E7 have their own specific ligand binding affinity and choice. Thus, this basic globin structure performs differently in each globin during various needs; it also depends on the temperature and pH due to the Bohn effect.

2.3.1.1 Similarity of neuroglobin to other globins Ngb has been studied in 11 mammals, 1 bird, and 4 teleost fish species (Burmester, Haberkamp, et al., 2007). The expressed sequence tags (ESTs) have shown that human Ngb and vertebral myoglobins share less than 21% of their amino acid sequences; human Ngb and vertebral hemoglobin share less than 25% of their amino acid sequences, which are greater than 1,100 protein when a comparison is made between man and mouse (Burmester, Weich, et al., 2000; Couture, Burmester, et al., 2001; Dewilde, Kiger, et al., 2001; Kriegl, Bhattacharyya, et al., 2002; Pesce, Dewilde, et al., 2003). Ngb has a high oxygen affinity with a half-saturation pressure (P50) of ~1.9-2.3 torr (1 torr at 37º C) when compared to mammalian hemoglobin (~26 torr), while myoglobin's P50 is even lower (~1 torr) (Burmester, T., B. Weich, 2000; Dewilde, S., L. Kiger, et al., 2001). Both Ngb and Cyto have several similar globin structures in intron/exon (3/4), disulfide bond (S-S), helices (α helices with A to H), the amount of globin (μM), cysteins, and structure.

Table 1. *The Biochemical and Physiological Characteristics of All Globin's Members*

Globins	Hemoglobin (Hb)	Myoglobin (Mb)	Cytoglobin (Cyto) Histoglobin (His)	Neuroglobin (Ngb)	GlobinX (Gb X)
Structure	Tetrameric	Monomeria	Monomeria	Dimeria	Unknown
Region (Helices)	A-H	A-H	A-H	A-H	A-H
Chromosome	Chrom11p15.5 Chrom16p13.3	Chrom22q13.1	Chrom 17q25.3	Chrom14q24.3	Unknown
Introns	2	2	3	3	2
Exons	3	3	4	4	2
Form	2α, 2β	1α, 1β	α	α	Unknown
Size	α: 141aa β: 146 aa 65 KDa	140 aa	190 aa 21KDa	151 aa 17KDa	200-205 aa 23 KDa
Amount	mM	mM	μM	μM	μM
Cysteins	CD7, D5	CD7, D5	CD7, D5, G18-19	CD7,D5, G18-19	Unknown
Oxygen affinity	26 Torr	1 Torr (highest)		1.5-2 Torr	Unknown
Characteristics	hexa: Fe^{3+}/ H_2O (high spin)	hexa: Fe^{3+}/H_2O (high spin) None	penta: normal hexa: chronic hypoxia/ischemia	penta: normal hexa: acute hypoxia/ischemia	Unknown
Disulfide bond (S-S)	None		Yes	Yes	Unknown
Location	RBC	Skeletal & heart muscles	All tissues Fibroblast of the ciliary & choroide Collagen synthesis	CNS, PNS, retina & endocrine	Low vertebrates invertebrates Fish & amphibians
Functions	O_2 binding, O_2 transport	Transport O_2 Scavenge NO	O_2 delivery Aerobic metabolism in the mitochondria Act as peroxidase	O_2 scavenger O_2 sensor Anti-hypoxia /ischemia protector Enzymes	Binding O_2 & gaseous ligands

2.3.2 Neuroglobin locations

Burmster, Weich, et al (2000) used an RNA Master Blot in the first study to quantify the Ngb expression through human and mice tissue. Ngb exists in neurons in both the central cerebral and peripheral neuronal systems, including the endocrine system and retina where nerve cells have high energy. In human brain tissue, the highest level of Ngb expression is found in the subthalamic nucleus. Expressed as a percentage of the level of expression in the subthalamic

nucleus, Ngb expression is 73% in the frontal lobe and the thalamus, 70% in the occipital pole, 61% in the medulla oblongata, 50% in the temporal lobe, 44% in the cerebral cortex, 43% in the substantial nigra, 21% in the putamen, 13% in the amygdale, 11% in the caudate nucleus, 11% in the hippocampus (CA1, 2, 3, and 4, especially in the pyramidal layer), 10% in the cerebellum, 23% in the whole brain (Wakasugi, Nakano, et al., 2003; & Saaler-Reinhardt, et al., 2002), and 34% in the fetal brain. Unlike this pioneering study, Zhang's later study (2002), which used Northern Blot analysis, showed that out of 12 human tissues only the brain showed Ngb expression. Cerebral cortex, hippocampus, thalamus, subthalamus, olfactory bulb, and brain cerebellum are the most important to cerebral function; they are also especially sensitive to cerebral hypoxia and ischemia and not surprisingly, show high Ngb mRNA expression in both mice and humans (Burmester, Weich, Reinhardt, & Kankeln, 2000, Zhang, Wang, Deng, Li, Wang, Fan, Xu, Meng, Qian, & He, 2002). Unfortunately, many Ngb studies use Ngb mRNA and protein from a variety of these locations, which may impair the ability to compare results.

Ngb expression has also been found in other human tissues such as the pituitary gland, appendix, adrenal gland, lungs, and colon. However, Ngb expression in the spinal cord, pancreas, small intestine, stomach, testes, lymph nodes, ovaries, and thymus is less than 10% than what has been found in the subthalamic nucleus. Ngb has not been found in the heart, aorta, skeletal muscle, bladder, uterus, prostate, thyroid glands, salivary gland, mammary gland, liver, spleen, peripheral leukocyte, lymph nodes, bone marrow, trachea or placenta, glial cells, heart, kidney, liver, spleen, thymus or fetal lung (Burmster, T., B. Weich, et al, 2000). Burmster first localized Ngb to the brain, but later researchers have found that Ngb has a high local concentration in neurons shortly after hypoxia and ischemia (Couture, Burmester, Hankeln, & Rousseau, 2001).

Generally, Ngb is found in the micromolar range and consists of less than 0.01% of the total protein content.

Schmidt et al. (2003) reported that the Ngb protein appears in all neurons of the animal retina (the plexiform layers and the ellipsoid region of the photoreceptor inner segment which consumes the most oxygen, and expresses Ng mRNA in the perikarya and the ganglion layers except pigment epithelium (Burmester, & Hankeln, 2004; Schmidt, Laufs, 2005; Bentmann, Schmidt, et al., 2005; Hankeln, Ebner, et al., 2005; Rajendram , Rao, 2007) but not in the retinal pigment epithelium. The concentration of Ngb in the total retina ranges from 100 to 200 μM, 100-200 fold greater than the total brain Ngb concentration, which is 1 μM (Dewilde, Kiger, Burmester, Hankeln, Baudin-Creuza, Aerts, et al., 2001; Schmidt, Giessl, et al., 2003). The retina has the highest oxygen demand of any tissue in humans. High metabolic energy regions require more energy and ATP from glycolysis and oxidative phosphorylation in terms of the functions of mitochondria and Ngb supplies in the retina. However, the concentration of Ngb in the eye may vary depending upon the proximity of the tissue layers to the mitochondria and major blood supply vessels.

2.3.3 The basic structure of neuroglobin (Ngb)

The human monomeric neuroglobin (Ngb) gene, which is located on chromosome 14q24.3, consists of 151 amino acids and has a small molecular mass of 17,000 Daton (17 kDa) (Burmester & Weich, et al., 2000; Dewilde & Moens, 2000; Sun & Jin, et al., 2001; Dewilde & Kiger, et al., 2001; Kriegl, Bhattacharyya, et al., 2002; Pesce, Nardini, et al., 2002; Wystub, Ebner, et al., 2004). Ngb has seven/eight α helices (A, B, C, D, E, F, G, and H), beginning with the N terminal and ending with the C terminal of the α-helics. The three intron/four exon

structure, located on the B12-2, E11-0, and G7-0 regions, are without genetic markers (Burmester & Weich, et al., 2000; Pesce, and Bolognesi, et al., 2002; Zhang, & Wang, et al., 2002; Pesce, & Dewilde, et al., 2003). The intron positions are coded as follows: B12-2 has position 2 of the 12th amino acid of the globin α-helix with 1445 base pairs (bp); E11-0 has position 631 bp; and G7-0 has position on 1,797 bp. The full-length cDNA of human Ngb according to the Gen Bank accession is 1,909 bp in size and contains an open reading frame from nucleotide 376 to nucleotide 831 (Zhang & Wang, et al., 2002). The 5'-non-coding region is 375 bp in length and the 3'-noncoding region is 1,078 bp in size. The Ngb has a genomic DNA sequence of 8,041 bp in size and three intons/four exons with a typical junctions GT-AG consensus motif of the splice donor and acceptor site. The Ngb contains 4753 protein atoms, 160 ordered solvent atoms, and seven sulfate anions (Pesce, Dewilde, et al., 2003). Figure 3 describes the structure of the Ngb in helices, intron/exon, and some special forms.

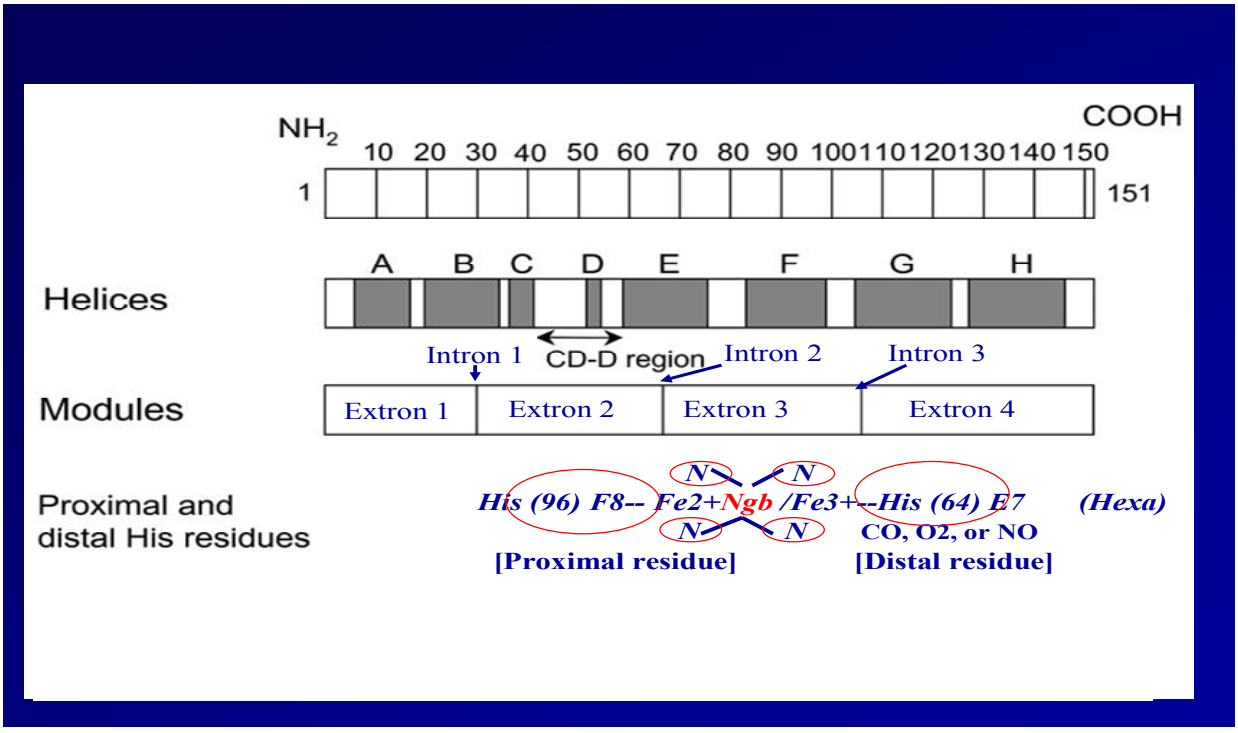


Figure 3. Summary of the Neuroglobin (Ngb) Structure

2.3.4 Special structures of neuroglobin (Ngb)

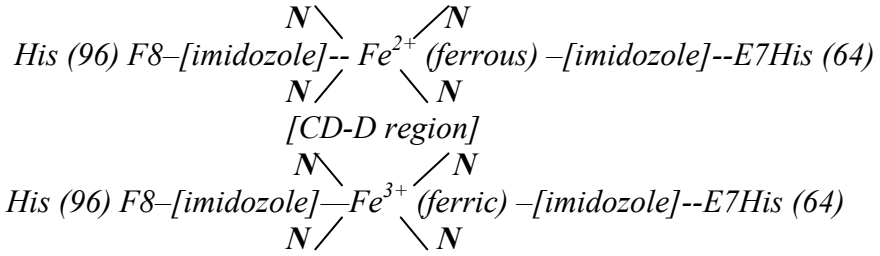
Ngb has five unique structures that maintain special functions in hypoxia or ischemia. The redox state for both Fe^{2+} and Fe^{3+} forms have pentacoordinated status and a hexacoordinated status. Secondly, the imidaoles stabilize the binding between heme (iron) with His64-E7 and His96-F8. Thirdly, the His64-E7 of the hexacoordinate state has biphases: high associated rate (K_{on}) with O_2 and carbon monoxide (CO) and low dissociation rate (K_{off}) with Fe^{2+}. Fourth, three cysteins (CD7, D5, and G18-19) and one disulfide bond (which exists in the extracellular space) are located at the CD and D of α-helix; these affect the O_2 binding action through oxidation and the reduction term on ferrous (Fe^{2+}). Finally, the CD-1 has a phenylalaine residue, which increases the protein-protein (π-π) interaction and influences the interaction in HisE7 and HisF8 with heme (iron) (Burmester, T., B. Weich, et al, 2000).

The iron atom in the heme prosthetic group of Ngb exists in either the Fe^{2+} or the Fe^{3+} redox with pentacoordinated (oxy) and hexacoordinated (deoxy) states (Reuss, S., S. Saaler-Reinhardt, et al., 2002, Pesce, A., S. Dewilde, et al., 2004). The Ngb presents the pentacoordinate under normal conditions, but becomes unstable and rapidly converts into ferric Ngb through autoxidation. The Ngb presents the hexacoordinate (low spin) under physiological states (anerobics or hypoxia/ischemia). The proximal (His96-F8) and distal (His64-E7) sites bind with heme (both Fe^{2+} and Fe^{3+}) on the range of helix E and F loop.

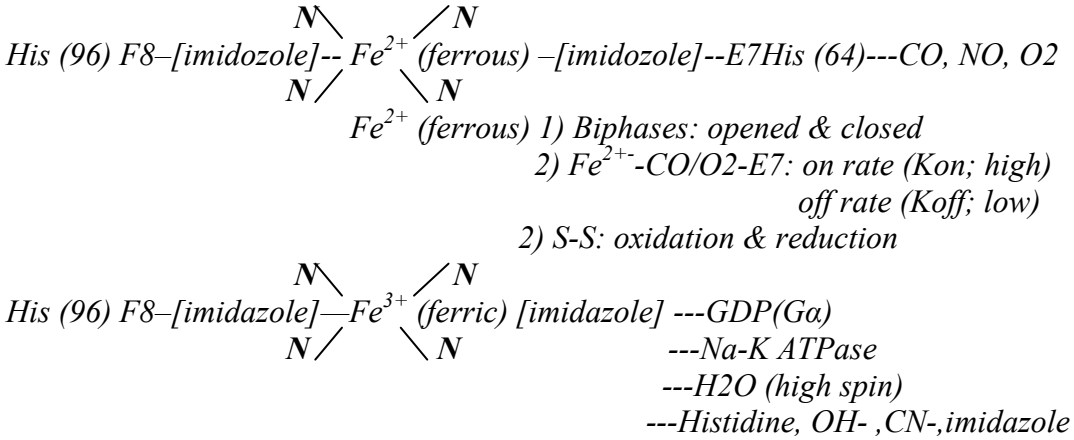
The proximal histidine (His96-F8) does not display any exogenous ligand but does show such endogenous ligands as water molecules (Kriegl, & Bhattacharyya, et al., 2002, Trent, 3rd and Hargrove, 2002, Hankeln, Ebner, et al., 2005). In the absence of the exogenous ligand, the Fe^{2+}/Fe^{3+} forms of hexacoordinated Ngb appear with the distal endogenous protein histidines (His64-E7). On the other hand, the distal histidine (His64-E7) dissociates coordinates to the

Fe^{3+}-Ngb center before an exogenous (external) ligand can bind to the heme; this mechanism controls the Ngb oxygenation. The largest cavity (120 Å) between His64-E7 and His96-F8 regions (CD-D) supplies the O_2 exchange (Pesce, A., S. Dewilde, et al., 2003). Thus, the large protein matrix cavity acts as a storage area for the ligand during the protein functional cycle in the hexacoordinated state. Figure 4 summarizes the Ngb structure in both the pentacoordinated and hexacoordinated states.

Pentacoordinated Ngb (oxygen, normoxic or aerobic, unstable)



Hexacoordinated Ngb (deoxyen, anerobics; stable)



ps: His96-F8 = proximal histidine (5^{th} coordinated);
His64-E7 = distal histidine (6^{th} coordinated)

Figure 4. Summaries of the Structure of Ngb in Both the Pentacoordinated and Hexacoordinated

The second unique structure of Ngb, the imidazole side chain of a histidine residue, is located at the fifth ligand, which is bound to the heme proximal His96-F8 histidine, and to the

sixth ligand at the distal His64-E7 histidine with an orthogonal azimuthal orientation in the Ngb (Couture, Burmester, Hankeln & Rousseau, 2001). The purpose of the imidazole is to stabilize the binding reaction between heme with HisE7 and HisF8. Distal His64-E7, which is directly, coordinated to the heme in both the Fe^{2+} and Fe^{3+} forms, causing a bend of the E-helix (Dewilde, Kiger, et al., 2001).

Third, O_2 and CO can display the distal histidine of ferrous Ngb to produce ferrous oxygen-bound Ngb (Ngb Fe^{2+}-O_2) or ferrous carbon monoxide–bound Ngb (Ngb Fe^{2+}-CO) (Wakasugi, Nakano, et al., 2004). When Ngb-Fe^{2+} binds with CO, the nearest residue has either a positive or a negative charge, which stabilizes the binding with CO. Nevertheless, when NgbFe^{2+} binds with CO, it has two states: closed when CO interacts with the residues; opened when there is no interaction. The binding capablities of O_2 and CO are similarly stronger as compared to the binding capability of NO_2. Within a few minutes, the average O_2 affinity of recombinant human Ngb becomes 1 torr at 37° C (Dewilde, Kiger, et al. 2001; Fago,. Hundahl, et al., 2004; Pesce, Bolognesi, et al., 2002; Geuens, Brouns, et al., 2003, Trent, Watts, et al., 2001; Dewilde, Kiger, Burmester, Hankeln, Baudin-Creuza, Aerts, et al., 2001). Remarkably, both O_2 and CO have a strong and rapid affinity, and do not easily disconnect from Ngb-Fe^{2+} in the hexacoordinated state. Kinetic studies on Ngb at room temperature (25-37° C) have found extremely fast CO and O_2 Kon to the pentacoordinate deoxy form (Kriegl, Bhattacharyya, et al., 2002). Histidine binding in the hexacoordinated takes about one millionth of a second. Koff times vary, depending upon the histidine affinity (as long as one second); oxygen binds rapidly but dissociates slowly (as much as one second) (Kiger, Uzan, et al., 2004). Furthermore, X-ray data of human Ngb (Trandafir, Van Doorslaer, et al., 2004) show that the affinity for exogenous ligands at positions B10, E7, and E11 is controlled by residues (O_2, NO, or CO). As a result, O_2

must compete with the proximal histidine to bind to the iron. Oxygen binding of Ngb may be linked to the redox state of the cell because O_2 is less sensitive than CO to electrostatic interactions and binds tighter with iron than CO. The following formula explains the relationship between CO and positive and negative residues in position HisE7.

$$NgbFe^{2+}\text{-}C{=}O\text{-}X+ \quad or \quad NgbFe^{2+}\text{-}C{=}O+X-$$

In addition, the expected rate of dissociation of Fe^{3+} from the His64-E7 of Ngb is lower in alkaline than in neutral conditions (Herold, Fago, et al., 2004; Vallone, Nienhaus, et al., 2004). Hexacoordinated Ngb-Fe^{3+} is associated with GDI (46 cystein and 53 cystein) and Na-K ATPase during hypoxia or ischemia. Ngb-Fe^{3+} has a seven-fold more stable bond with cyanide (CN-) in the presence of this disulfide bond (S-S) in alkaline conditions. Moreover, it has high spin binding with water molecules and low spin binding with carbohydrate (OH-), histidine, imidazole, and cyanide (CN-) in the absence of cystein S-S bonds. No polar or low polarity residues or ordered water molecules are present inside the heme distal pocket (Pesce, Dewilde, et al., 2003).

Ngb has three cysteine residues (position in CysCD5 (46), CysD7 (55), and CysG18-19 (121) which form intracellular disulphide bonds (S-S) between the CD and D loops (Pesce, Dewilde, et al., 2003; Hamdane, Kiger, et al., 2003; Vinck, Van Doorslaer, et al., 2004; Hankeln, Ebner, et al., 2005; Hankeln, Ebner, et al., 2005). The cystein residues in globins form disulfide or catalytic bonds by being part of direct ligand binding. The disulphide bonds contribute to oxygen affinity under certain pH (4-11) and temperature (25°-37°C) conditions. A stable structure of the disulfide bond between Cys46 and Cys55 of Ngb is located at the CD and D helix in hypoxia (Wakasugi, Nakano, et al., 2004). Thus, either the Fe^{2+} with sulfur-bridges human Ngb or Fe^{3+} with O_2 affinity reduces the histidine binding affinity, but does not directly

influence protein stability. Almost 90% of the O_2 affinity occurs with the disulfide bond reduced under anaerobic conditions (Hamdane, Kiger, et al., 2003; Trandafir, Van Doorslaer, et al., 2004). These anaerobic conditions reduce the disulfide bridges in human Ngb and allow the Ngb to bind with oxygen.

Finally, the CD-1 phenylalaine enhances the protein-protein (π-π) interaction between heme with His96-F8 in both pentacoordinated and hexacoordinated forms. The cystein exists in the CD-D region, which is especially important in hypoxia. The π-π reaction in the human Ngb is not only on a certain area but is also involved with oxygen binding.

2.3.5 Comparing Ngb in humans and rats or mice

Several basic identified structures have been found in Ngb in humans and mice. The following table explains the difference in molecular mass, helix chains, intron and exon structure, cavities, cystein and histidine, molecular, cDNA, nucleotic acide sequence, and amino acid sequence. Table 2 compares Ngb in Humans and Rats or Mice.

Table 2. Comparison of Ngb in Humans and Rats, or Mice

Characteristic	Human	Rats/ Mice
Mass of molecule	17 kDa (16.931 w S-S; 16,930 w/o S-S)	18 kDa
Chromosome	14q24.3	14q24.3
Helices chain	1α	1α
Intron	B12-2. G7, E11	B12-2, G7, E11
Disulfide bond (S-S)	Yes	Yes
Structure	His(64)E7-Fe-His(96)F8	His(64)E7-Fe-His(96)F8
Cavity	1 matrix cavity (120 Å)	2 small cavity (16Å, 11 Å) 1 large (287 Å)
Cystein	CD7, D5, G18-19	CD7, D5
Molecular	CD-D flexibility conformation 4753 protein atoms 160 ordered solvent atoms 7 sulphate anions	EF 3-148 amino acid 116 water molecular
cDNA	1909 base pairs (bps)	
Nucleotic acid sequence		Rat: 86% similar to mouse 88% similar to human
Amino acid sequence	151 aa	159 aa Rat: 96% similar to mouse 94% similar to human

2.3.6 Roles and functions of neuroglobin

Researchers have shown that the Ngb has the following functions: 1) a scavenger NO; 2) an enzyme with nicotinamide adenine dinucleotide hydrogenase NADH oxidases for glycolysis from ATP; 3) an O_2 carrier or sensor that diffuses to mitochondria under aerobic or anaerobic metabolism and transports O_2 concentrated response; 4) a detoxifier; and 5) a neuroprotector from ischemic insult and apoptosis through cellular death receptors or pathways (Moens, Dewilde, 2000; Dewilde, Kiger, et al., 2001; Goovaers, et al, 2002; Reuss, Saaler-Reinhardt, et al., 2002; & Van Doorslaer, S., S. Dewilde, et al., 2003; Wakasugi, K., T. Nakano, et al., 2003; Burmester, & Hankeln., 2004; Fordel, Geuens, et al., 2004, Trandafir, Van Doorslaer, et al.,

2004; Hankeln, Ebner, et al., 2005; Schmidt, Laufs, et al., 2005; Fordel, Thijs, et al., 2006). Animal models have provided a better understanding of Ngb expression and its functions by demonstrating Ngb expression and function in neuronal hypoxia *in vitro* and focal cerebral ischemia *in vivo* (Sun, Xiao, Zhu, Greenberg, 2001).

Ngb, which is induced under hypoxic/ischemic and oxidative stress conditions, protects neurons (Pesce, Dewilde, et al., 2003). However, under hypoxic or ischemic conditions, Ngb transports oxygen across the BBB and increases the availability of oxygen to mitochondria of highly metabolic neuronal tissues (Burmester, T., B. Weich, et al, 2000). These previous studies show that Ngb, in order to reach the BBB and endothelial cells, is located close to the mitochondria in neurons, as well as close to the cellular surface. Thus, Ngb is sensitive to the biochemical changes in cells at the first acute moment of crisis.

2.3.6.1 Neuroglobin (Ngb) as a NO scavenger Ngb acts as scavenger of both reactive oxygen and nitric oxide species. Both nNOS and eNOS are calcium dependent and located on chromosome 12 and chromosome 7 (Lipton, 1999). The reasons that Ngb may be better than nNOS during hypoxia and ischemia are that Ngb 1) does not rely on calcium influx into the cells, 2) does not cause vasodilatation; and 3) does not produce any toxicity in cells (Leker, & Shohami, 2002). As a consequence, Ngb is better pathway than nNOS to protect against hypoxia and ischemia.

Ngb serves to save cells from DNA breakdown, endoplasmic reticulum (ER) disturbance, loose glucose, lipid, protein synthesis, and biochemical damage (Fordel, Thijs, et al., 2007, Burmester, & Hankeln, 2004, Van Doorslaer, Dewilde, et al., 2003). Researchers have found that only Ngb- Fe^{3+} is associated with all toxic products from ROS and NOS by binding with NO and

releasing more oxygen to supply the needs of the anaerobic metabolic state in order to decrease the levels of cytochrome c, calpain, and caspase in mitochondria. Ngb-ferryl (Ngb-Fe^{4+}) has no association with ROS and NOS (Fordel, Thijs, et al., 2007). Therefore, Ngb-Fe^{4+} cytotoxicity does not interact with peroxides (Fago, Hundahl, et al., 2004). The Ngb in its NgbFe^{2+}-NO (nitric oxide bound) form is an efficient scavenger of $ONOO^-$, which is generated at a high rate following brain ischemia. Thus, Ngb binds with all ROS and NOS in order to release O_2 into the mitochondria in the cells; this reverses the pathway to apoptosis and necrosis (Figure 5). Rodrigo et al reported that Purkinje cells and dendrites expressed nNOS and iNOS with the Ngb after finding ischemia-reperfusion (2001).

Ca++ (up; depolarization):
→ROS ----- (superoxide dismutase) -->H2O2 (hydrogen peroxide)
----- (cytosol) ----------→ O2- (superoxide)
→ NOS (iNOS, eNOS, & nNOS)
→ NO + O2-(superoxide) → ONOO- (peroxinitrite)
ONOO-+ CO2 --> ONOOCO2- (nitrosoperoxocarboxylate)
→NO (mitochondria) → cytochrome C → calpain →caspoase 3/7→ DNA fragment→ cell death

Figure 5. Ngb vs. ROS and NOS

2.3.6.2 Neuroglobin (Ngb) as an enzyme This section describes each special enzymatic pathway of Ngb in hypoxia at the ischemia level. Ngb is an enzyme which reacts with soluble granulate cyclase-protein kinase G (sGC-PKG), protein kinase C (PKC), cyclic guanosine 3', 5'-monophosphate (cGMP), guanine nucleotide dissociation inhibitor (GDI), regulators of G signaling (RGS), and G protein-coupled receptor kinases (GRK), cytochrome c, flotillin, cathepsin, cysteine, and hippocampus neurons 33 (HN33). This section presents the special pathways and enzymes with Ngb in hypoxic and ischemic conditions.

Soluble guanylate cyclase-protein kinase G (sGC-PKG), in Purkinje cells of the cerebellum and olfactory bulb, as well as protein kinase C (PKC) pathways are involved in hemin-induced gene expression and erythroid differentiation (Figure 6) (Zhu, Sun, Jin, & Greenberg, 2002). Both sGC-PKG and PKC are heme gene expressions that induce and react with Ngb to protect cells during hypoxia and ischemia. As a part of the heme gene, cGMP is a fundamental intermediary in the photo transduction response and synthesis. cGMP is catalyzed by the enzyme guanylate cyclase, which is activated by NO. High levels of guanylate cyclase have been detected in Purkinje cells of the cerebellum and in the olfactory bulb, where the Ngb is expressed during hypoxia and ischemia (Fago, Hundahl, et al., 2004).

Hypoxia/Ischemia → sGC-PKG, PKC, and cGMP (*heme genes) → *Ngb (up)

Figure 6. Protein G vs. Ngb

Under hypoxia/ischemia, Ngb brings more O_2 to stop DNA fragmentation in mitochondria due to cytochrome c, caspase, and calpain toxicity (Figure 7.) While cathepsin is toxic in the intracellular space, cystain c and flotillin works with Ngb in order to decrease cell death.

Aerobics: Cytochrome c (down) → caspase (up) → calpain (up) → DNA fragment → cell death

Cytochrome c (up) ←---------- Ngb (mitochondria)

Hypoxia or ischemia: Cathepsin (up) from lysosome ←-----Cyctatin C and Flotillin + Ngb (up)

Figure 7. Under Aerobic and Hypoxic Conditions and Neurochemical Mechanisms

The four peptides of the human Ngb (Ngb 31-47, Ngb 48-66, Ngb 48-67, Ngb 103-119) are located at the binding site for Gα (Zhu, Sun, Jin, & Greenberg, 2002). The human Ngb Fe^{3+},

a result of rapid autoxidation, binds exclusively to the GDP-bound form of the Gα and frees the DTP with Gβγ under a stress-responsive sensor for signal transduction in the brain (Nienhaus, Kriegl, et al., 2004; Vallone, Nienhaus, et al., 2004; Sun, Jin, et al., 2003; Rajendram, 2007; Wakasugi, K., Nakanoet al., 2003). As a result, Ngb delivers O_2 and NO as a guanine nucleotide dissociation inhibitor (GDI) which functions as the Gβγ and leads to protection against neuronal death (Anselmi, Brunori, et al., 2007; Khan, Sun, et al., 2007; Kitatsuji, Kurogochi, et al., 2007; Kitatsuji, Kurogochi, et al., 2007; Burmester, & Hankeln, 2004; Hankeln, Ebner, et al., 2005; Schwindinger, & Robishaw, 2001). Consequently, human Ngb has a 25 - 35% amino acid sequence homology with regulators of the G protein signaling (RGS) on extron and assists protein-protein interaction between the Ngb Fe^{3+} and GDI (GDP-Gα). This suggests that oxidized Ngb (Fe^{3+}Ngb) inhibits GDP released from the Gα protein. Acidity, which decreases the rate of exogenous ligand binding to the pentacoordinate species, favors hexacoordination. Nevertheless, the coupled conformational change involves the EF loops of the Ngb.

The on/off G protein ratio is either a ligand or signal-activated G protein-coupled receptor (GPCRs) that induce a GDP release from a Gα subunit and bind to the GTP (on) or GTP dissociated GPCR and Gβγ by biding with Gα (off) (Figure 8). The activity of the on/off G protein is based on a) guanine nucleotide exchange factors (GEFs), which stimulate GDP dissociation and subsequent GTP binding; b) guanine nucleotide dissociation inhibitors (GDIs), which inhibit GDP dissociation; and c) GTPase-activating proteins (GAPs), which enhance GTP hydrolysis (Wakasugi, K., T. Nakano, et al., 2003). Gβγ stimulates proliferation via a mitogen-activated protein kinase (MAPK) pathway and promotes cell survival by activation of phosphotidylinositol 3-kinase (Wakasugi, K., T. Nakano, et al., 2003).

Aerobics: GDP (Gα) (Gβγ) ----------GPCR-------→ GTP (Gα) & GTP (Gβγ)
Anaerobics: GDP (Gα) (Gβγ) ---------- ---→ GDP (Gα) & G (βγ) +NgbFe3+

Figure 8. GDP in Aerobics and Anaerobics

2.3.6.3 Neuroglobin (Ngb) as an O2 sensor The brain only contains 2% of the body's total weight; however, during rest it consumes about 20% of the available oxygen (Burmester, Weich, Reinhardt, & Hankeln, 2000; Zhang, Wang, et al., 2002; Wakasugi, Nakano, et al., 2003). Hence, Ngb plays a major role by transporting oxygen across the BBB, increasing the metabolic oxygen in neuronal tissues. Both the pH (acid or alkaline Bohr Effect) and temperature-dependent O_2 affinity influence O_2 and CO binding on the Ngb (Fago, A., C. Hundahl, et al., 2004). This principle is based on the theory that when O_2 decreases, CO_2 increases; inhibiting decreases in pH.

Based on the high K_{on} and low dissociation rates K_{off}, the intrinsic affinities for O_2 and CO for the pentacoordinated form are quite high (Dewilde, S., L. Kiger, et al., 2001). At room temperature, the Ngb has K_{on} and K_{off} rates for O_2 and CO below a pH value of 5-10 (Couture & Burmester, 2001; Pesce, Nardini, et al., 2002). Whenever the distal site is free (pentacoordinated form), a high rate of binding occurs; whenever the external ligand encounters the hexacoordinated form, the histidine dissociates, and the overall ligand is slowly replaced. When the Ngb is under acidosis, the rate of exogenous ligand decreases binding to the pentacoordinate species (normoxia), favoring hexacoordination; therefore, the coupled conformational change involves the EF loop (Vallone, Nienhaus, et al., 2004).

In addition, a low O_2 level causes an increase in NADH and facilitates the glycolytic production of ATP under semi-anaerobic conditions (Figure 9). It also reduces the cysteines,

breaks the disulfide bridge, and changes the release of O_2 from the Ngb (Burmester, and Hankeln, 2004; Hankeln, Ebner, et al., 2005; Pesce, Bolognesi, et al., 2002). NO blocks all mitochondrial iron-sulfur enzymes, including NADH, which plays a major role for the Ngb by keeping the heme iron atom in an Fe^{2+} form during normal status (Wink, & Mitchell, 1998, Geuens, Brouns, et al., 2003, Trandafir, Hoogewijs, et al., 2007, Kitatsuji, Kurogochi, et al., 2007). Conversely, when the O_2 concentration increases, the free cysteines oxidize into an intramolecular disulfide bond with the O_2 (Hamdane, Kiger, et al., 2003). On the other hand, when hypoxia/ischemia manifests in the neurons, NADH+ reduces the process in the citric cycle as well as the disulfide bond of the Ngb. The disulfide bond changes the histine binding affinity with reduction (oxygen released) and oxidation (without oxygen released) mechanisms.

Hypoxia/Ischemia NO (up) --------→ NADH <----------Ngb (up) [O2-HisE7-Fe2+-HisF8]
Ca++ (up)

Figure 9. Hypoxia/Ischemia Ngb vs. NADH

2.3.6.4 Neuroglobin as a detoxifier Toxifers, at some stage in hypoxia or ischemia, include the following: 1) the substances from ROS and NOS (H_2O_2, NO, ONOO-, CO_2, $ONOOCO_2^-$); 2) cathepsin, cobalt chloride ($CoCl_2$), and deferixiamine (Dfx) or sodium nitroprusside (NaNP); and 3) these hypoxia or ischemia inducible receptors or pathways: HIF, MAPK, NMDA, AMPA (Figure 10.). Therefore, $NgbFe^{2+}$ tries to carry O_2 against the surrounding environment without hurting the function of the cells and each organism.

Aerobics: *NOS <---cytochrome c ←--- caspase ←----- calpain <-----DNA fragment*

(iNOS, nNOS, eNOS)

Cathepsin, cystatin C, flotillin in lysosome (normally)

NMDA, MAPK, HIF, NADH (stable)

Anerobics: *NOS ---NO→ cytochrome c --→ caspse ----→ calpain --←-- $NgbFe^{2+}$-O_2*

(mitochondria)

NO+ O_2- →ONOO- or O_2+CO_2- →ONOOCO_2- ←- $NgbFe^{2+}$-O_2

(cellular)

NO→ blood vessel →vasodilation and blood flow decreased

Glucose (down)→ glycolysis →NMDA

MAPK→ GTP ←----$NgbFe^{3+}$-GDI

O_2 (dropped) ----------→MAPK→HIF → Gβγ←----$NgbFe^{3+}$-GDI

NADH (mitochondria) ←----$NgbFe^{2+}$-O_2

Figure10. Comparing Aerobics and Anerobics in NOS, O2, & Glucose Reaction

2.3.6.5 Neuroglobin as a neuroprotector from hypoxia or ischemia insult There is only limited knowledge concerning the role of Ngb in brain hypoxia and ischemia (Figure 11) Throughout hypoxia or ischemia, mitogen-activated protein kinase (MAPK) stimulates the GTP and likewise indicates the HIF to the Gβγ (Hankeln, Ebner, et al., 2005, Sang, et al. 2003). HIF-1 binds to the hypoxia response element (HRE) in the human EPO gene (5'-TACGTGCT-3'), which is identified by encoding (5'-RCGTG-3'). This gene sequence is shared by vascular endothelial growth factor (VEGF), heme oxygenase (HO), iNOS, glucose transporter-1 (GLUT-1), glycolytic enzymes aldolase A, enolase 1, lactate dehydrogenase A, phosphofructokinase L, and phosphoglycerate kinase I (Fordel, Geuens, et al., 2004, & Rajendram R, Rao NA 2007). HIF-1 is a phosphorylation-dependent and redox-sensitive protein that binds DNA in the major channel. While the HIF-1α is induced by hypoxic/ischemic stress to produce more Ngb, it also inhibits the MAPK/extracellular signal-regulated kinase (MEK) which activates HIF-1 (Pesce, Bolognesi, et al., 2002; Burmester & Hankeln, 2004; Wystub, Ebner, et al., 2004). To sum up, when hypoxic/ischemic injury to neurons or neuroprotective proteins occurs, Ngb reacts with the

EPO, VEGF, and heme oxygenase increases in anaerobic metabolism, tissue, vascularity, and oxygen delivery to maintain neural cell survival (Sun, Jin, et al., 2001).

Hypoxia-------→ HRE – HIF 1α -- MAPK, MEK----------→Ngb (decreases)
(Gene: related to: ----------→ Ngb (increases)
VEGF, HO, iNOS, Glut-1, aldolase A,
lactate dehydrogease A, phosphofructokinase L, phosphoglycerate kinase

Figure 11. Neuroprotectors under Hypoxia

The sodium-potassium pump (Na^+/K^+ ATPase), located in the plasma membrane, maintains its resting potential while reacting with NaNP during hypoxia or ischemia. Accordingly, when the Ngb plays as the GDI in anaerobic, the Na^+/K^+ ATPase follows a similar pathway to the Ngb in the intracellular space.

2.4 POSITIVE AND NEGATIVE NEUROGLOBIN FINDINGS IN ANIMAL AND HUMAN MODEL

Under hypoxia/ischemia, the lack of oxygen stimulates the Ngb genes; Ngb mRNA was detected in the cytoplasm of neurons in many brain regions following hypoxia or ischemia in mice (Reuss, Saaler-Reinhardt, et al., 2002). Hypoxia/ischemia is known to reduce the pH and temperature of neuronal cells (Brunori, & Vallone, 2007; Fordel, Thijs, et al., 2007). This section focuses on those studies, using both animal models and humans, with both positive and negative outcomes.

Sun, Xiao, Zhu, and Greenberg (2001) experimented with focal cerebral ischemia by occlusing the middle cerebral artery (MCAo) for 90 minutes, followed by reperfusion for 4-24 hours. This led to greater Ngb immunoactivity in the cytoplasm of neurons around the ischemic

penumbra and less in those found in the ischemic core. In the same year, Venis also reported that an overexpression of the Ngb caused less hypoxic injury in mouse cells.

Zhu, Sun, Jin, and Greenberg, (2002) discovered that hippocampus neurons 33 (HN33) induced Ngb expression. They used Western Blot to show an increase of four times over normal in the expression of Ngb protein within two hours. After 3 days, Ngb expression returned to normal.

Sun, Y., Jin, K., Alyson, P., Xiao, O.M., Lin, X., Greenberg, D.A (2003) induced MCAo in Sprague-Dawley rats for 90 minutes and reperfusion for 24 hours. They discovered that a greater quantity of Ngb was associated with a 56-60% decrease in infarct volume ($p < 0.02$, N = 5 per group). Moreover, they found no difference among the venous blood concentration of Na^+, K^+, Ca^{2+}, and HCO_3-or hemoglobin arterial blood pH, PO_2, or PCO_2, or mean arterial blood pressure.

The following human Ngb studies are based on the work of Rajendram and Rao (2007). After studying three glaucoma patients (ages 3, 67, and 69 years), they discovered that the outer plexiform, inner segment of the photoreceptor layer, and inner/outer nuclear layers, but not the inner plexiform and ganglion cell layer, had the same positive Ngb expression and mRNA Ngb as found within the normal human retina associated with mitochondria. When intraocular pressure increased to pathological glaucoma levels, blood flow did not rise, but the oxygen needs did. However, Ngb expression in humans has not yet been demonstrated under natural conditions. While Ngb may serve to limit brain damage within a stroke population (Venis, 2001), no study has yet proven a correlation between Ngb function and ischemia in humans.

Using an animal model, Zivic (1998) found that the average time to Ngb expression began in an average of 19.1 minutes within the cortex in response to half-maximal ischemia

damage, compared to 12.7 minutes in the hippocampus. He subsequently found a four times greater concentration of Ngb in the cortex than in the hippocampus. The study shows that the hippocampus might have less Ngb expression than the cortex. In other experiments, Greenberg and his colleagues cultured mouse neuronal cells with and without O_2 for 24 hours and found the experimental group had a higher level of Ngb protein and mRNA than the controls (Venis, 2001). Neuroprotection from *in vivo* ischemia, decreased by administration of an Ngb antisense oligodeoxynucleotide, is enhanced by Ngb overexpression (Sun, Jin, et al., 2001; Zhu, Sun, et al., 2002; Garry, & Mammen, 2003; Garry, Mammen, Pradeep 2003). Ngb expression increased under hypoxic conditions *in vitro* as well as during focal cerebral ischemia *in vivo* (Sun et al., 2001). These study results explain the effects of Ngb concentration on infarct size (50% decreased) and functional deficits after a 90-minute occlusion of the middle cerebral artery in a rat model during the reperfusion period. An Ngb antisense oligodeoxynucleotide (ODN) increases infarct size two-fold; it also worsens the neurological outcome after an induced *in vivo* focal ischemia in the rat following treatment with an adenoma-associated virus (AAV) targeted to the Ngb (Sun, Jin, et al., 2003). Sun, Jin, et al. (2001) and Brunori, Giuffre, et al. (2005) tested Ngb detoxification of NO by catalyzing the reduction of 2NO to N_2O in vitro with a negative outcome.

Shan et al. (2004), who used the gerbil model of global forebrain ischemia, found that increased levels of Ngb and its mRNA were expressed within 10-20 minutes. In 2000, Greenberg and his colleagues researched mouse neuronal cells both with and without O_2 for up to 24 hours. In another study in 2001, Ngb mRNA and protein upregulated 2.5 fold after 16 hours of anoxia in the animal model. Cells deprived of oxygen produced higher levels of Ngb protein and mRNA than the controls. *In-vivo* simulation of ischemia by cerebral vascular occlusion for over 90

minutes also resulted in a higher expression of Ngb in rat cortex cells (Venis, 2001). Ngb not only protects the brain but also uses the high level of the eNOS within endothelial cells to reduce infract volume in cardiac tissue (Khan, Wang, et al., 2006). Sun Jin, et al. (2001) deprived cells of O_2 for up to 24 hours; this caused a greater than two-fold increase in Ngb and its mRNA which related to the hypoxia inducible pathway and the addition of CoCl or deferoxamine. The same results were found by Zhu and Sun., et al (2002) study.

Casado, Pannell, Whalen, Clauw, and Baraniuk, J.N. (2005) took CSF from six humans with chronic pain. They studied six different neuroglobin peptides by investigating the mass over-charge (m/z), charge state, elution time, position, sequence, and molecular weight for each peptide by using a CapLC nanoESI Q-TOF tandem mass spectrometry. CSF still provides 60% of total brain neuroglobin mRNA expression. This study found no association between the presence of neuroglobin and such clinical factors as age, duration of pain, and tenderness to pressure.

In the study of Shang, Zhou, Wang, Gao, Fan, Wang, et al. (2006), Ngb expression was found in serum in following sham occlusion 2, 8, 48, and 72 hours (1.42-9.2ng/ml ($M \pm$ SD=4.61$\pm$ 2.53 ng/ml in sham; 5.82$\pm$3.5; 7.69 $\pm$2.97 ng/ml, 2 fold, and 3 fold) after 20 minute bilateral common carotid artery occlusion in Mongolian gerbils. The authors explained that ischemia insult impairs the BBB and induces neuronal membrane dysfunction. The Ngb protein then appeared in the blood serum and cerebral spinal fluid which Casado reported in his 2005 article. Mammen and Shelton, et al. (2002) demonstrated increased Ngb expression by using 10% O_2 and 90% N_2 for one hour in adult murine mice. The positive effects of Ngb contributed to the survival of the tissue in the penumbral area around the occluded vessel. This zone has a very low concentration of O_2 but a higher level of NO (Brunori & Vallone 2006).

In contrast, some studies of animal models, including that of Hundahl and Kelsen, et al. (2006), found negative results regarding Ngb and its mRNA in response to ischemia. These studies reported that less Ngb mRNA was expressed in the ischemic hemispheres of transient MCAo animals after 24 hours ($p \leq 0.002$). A lower number of Ngb ($p \leq 0.004$) and NeuN-positive ($p \leq 0.001$) striatal neurons were found in transient MCAo rats, but the Ngb was found in the limbic system using immunohistochemistry. Less than 1/2,500 of neurons in the striatum expressed Ngb and there was a strong negative correlation ($\gamma = 0.85$; $p \leq 0.02$) between Ngb immunopositive neurons and infract volume. Mammen and Shelton, et al. (2002) reported that their study showed no Ngb mRNA in the brains of mice after prolonged hypoxia (up to 14 days with 10% O_2). Mammen and Shelton, et al. (2002) did not succeed in detecting Ngb expression in two week chronic 20% O2 / 90% N_2 using situ hybridization with a radioactive probe. Sun, et al. (2001) demonstrated an acute hypoxia model in cortical neurons with 95% N_2+5% $CO_2 \leq 24$ hrs, which only revealed HIF.

Several studies have described Ngb mRNA or protein in humans and animals. High levels of mNRA and protein expression, particularly in the basal ganglia, cerebral cortex, hippocampus, and cerebellum, relate to age, neurological disorders (e.g. hypoxia in stroke) and neurodegenerative diseases (oxidative stress, Alzheimer's disease, Huntington's disease, and spinocerebellar ataxias) (Sun, Jin, et al., 2005). In rats, Ngb expression decreased with age after 6-8 weeks; further changes occurred in 3 (> 80% in parietal neocortex), 12, and 24 (< 10% in temporal neocortex and hippocampus) month old Sprague Dawley rats. In another study, Schmidt-Kastner et al. (2006) found that Ngb mRNA was bound in the control group for the first 10-20 minutes, indicating that it existed for the first 30 minutes ($\rho < 0.05$).

The results of these studies are summarized in the tables below, with studies summarizing positive findings in Table 3, and studies with negative findings in Table 4.

Table 3. *Summary of POSITIVE Ngb Expression Studies*

Published year, place, & authors	Published purpose, subjects, experimental method, time, & tissue	Results
Burmester, T et. al 2000, USA	S: Mouse and human Ngb cDNA genomic region of the mouse and human gene E: Expressed sequence tags (ESTs), northern hybridization for RNA, RT-PCR	Ngb RNA(+): frontal lobe, subthalamic nucleus, thalamus in mouse brain Ngb human tissue (+): expression level
Zhang, C et.al 2001, China	S: Human total brain mRNA (1μg) and sequence of human Ngb Male adult Wistar rats (200-250g) Ngb gene expression E: Northern blot	Ngb in human tissue (+): brain Ngb in rats (+): m RNA in cortex, hippocampus, forebrain, thalamic nucleus, hypothalamic area, cerebellum, and brain stem
Schmidt, M et. Al 2001, Germany	S: Mice E: Western blot, immunostaining, in situ hybridization	Ngb (+): retina with mRNA expression
Sun, Y., et al. 2001, USA	S: Mouse, in cerebral neurons (HN33) E: 90' MCAo followed 4-24 hours reperfusion Western Blot/Cytochemistry/Immunocytochemistry Oligodeoxynucleotide (ODN) treatment	Ngb in cytoplasm Ngb in penumbra and not in ischemia
Mammen, P. P., 2002, USA	S: Adult murine brain E: Chronic 10% O_2 + 90% N_2 * 1 hr. 10% O_2 for 2 wks situ hybridization (ISH), RT-PCR/ Microscopy & photomicrography	corenormoxic adult mouse: Ngb (+) in focal regions of the brain, including the lateral segmental nuclei, the peptic nucleus, amygdale, locus coeruleus, and nucleus of the solitary tract.
Zhang, C., 2002, USA	S: Human, rat, and mouse Ngb E: Bioinformatic analysis/the rapid amplification of cDNA ends (RACE) / degeneracy PCR/in situ hybridization, & immunohistochemical	Human Ngb: 1909 bp in size and the genomic sequence is 8041 bp in size (GenBank Accession No. AF422797). Adult rat brain: cerebral cortex, hippocampus, thalamus, hypothalamus, olfactory bulb, and cerebellum. normal adult rat brain: cerebral cortex, hippocampus, thalamus, hypothalamus, pons, and cerebellum

Note: S=subject; E=experimental methods

Table 3. *Summary of POSITIVE Ngb Expression Studies (Continued)*

Published year, place, & authors	Published purpose, subjects, experimental method, time, & tissue	Results
Zhu, Y., 2002, USA	S: HN33 E: RT-PCR and Northern Blot/Western Blot	Ngb & Ngb mRNA increased at 8-24 hrs the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase kinase inhibitor protein kinase G and soluble guanylate cyclase G (sGC-PKG)
Sun, Y. et al. 2003, USA	S: Adult rats E: 90' MCAo (50% decreased in size of cerebral infarct) followed 72 hrs.	60% cerebral infract volume increased
Geuens, E., 2003, USA	S: Mouse Ngb & Cyto E: In situ hybridization/immunocytochemistry/ELISA Western Blot	Ngb (+): intensities and in the islet of Langerhans in the pancreas.
Schmidt, M. 2003, USA	S: Mouse retina E: Gel electrophoresis & Western Blot	Ngb (+): in all neurons of the retina but not in the retinal pigment epithelium. Ngb mRNA (+):the plexiform layers and in the ellipsoid region of photoreceptor inner segment, perikarya of the nuclear and ganglion layers of the neuronal retina,
Sun, Y., 2003, USA	S: Sprague-Dawley rats (290-320 g); n= 5 each group E: Focal cerebral ischemia (90 min MCAo followed 24 hrs) Ngb antisense oligodeoxynucleotide (ODNs), Western Blot, immunohistochemistry	49-52% cerebral infract volume increased ($p<0.02$) No differences in venous blood, Na+, K+, Ca2+, HCO3-, and Hb in arterial pH, PO2, PCO2 or MBP
Wystub, S., et al. 2003, Ireland	S: In neurons of the mouse brain. E: In situ hybridization/ ELISA/preabsorption tests immunohistochemistry.	Ngb-mRNA (+): in neuronal cells of the central and peripheral nervous systems as well as in endocrine cells. Ngb protein: cerebral cortical regions,

Note: S=subject; E=experimental methods

Table 3. *Summary of POSITIVE Ngb Expression Studies (Continued)*

Published year, place, & authors	Published purpose, subjects, experimental method, time, & tissue	Results
Fuchs, C., 2004, USA	S: D.rerio Ngb c DNA & gene E: Flash photolysis kinetics , Western blotting, immune staining, and mRNA in situ hybridization. Cell fractionation, Western Blot	subcortical structures such as thalamus and hypothalamus, nuclei of cranial nerves in the brainstem and cerebellum Ngb (+): in the fish central nervous system and the retina but also in the gills, the chloride cells
Hundahl, C. et. al 2005, Belgium	S: Female mice E: Hypoxia (mixed O_2[7.6%]+N_2) * 2hrs RT-PCR	Ngb (+): cerebral cortical, thalamus and hypothalamus nucleus, plexus choroideus, olfactory bulb, brainstem
Casado, B. et. al 2005, USA	S: CSF of chronic pain humans E: Mass spectrometry	Ngb(+) 6 peptides: in CSF
Sun, Y. et. al 2005, USA	S: Rodent brain E: Western Blot, RT-PCR	Ngb (+): cortex, striatal and hippocampus neurons, cerebellar Purkinje cells Ngb (-): atrocities
Shang, A., et al. 2006, Netherland	S: Male Sprague-Dawley (172-225 gm) brain cortex E: Either sustained hypoxia (SH; 10% O_2) or intermittent hypoxia (IH; 10% and 21% O_2 alternating every 90 s) for 1, 3, 7 and 14 days, Immunoreactivity/Quantitative real-time RT-PCR	SH: Ngb mRNA and protein (+) IH: slight in Ngb expression at day 1(+)
Shang, A. et. al 2006, USA & China	S: Gerbil E:Global cerebral ischemia (20')-reperfusion with 2-72 hours Immunocytochemical	
Hunhahl, C. et. al 2006, Denmark	S: Rats E: MCAo (90') and 1 wk	Ngb (+): 8 & 48 hours peak point Cortex, hippocampus Ngb mRNA (+): after 24hrs & 1wk (P<0.00) on MCAo group
Rajendram, R. et. al. 2007, USA	S: 3 human Glaucoma patients E: Western blot	Ngb expression: cortex, hypothamus, amygdale, & striatum Ngb (+): photoreceptor inner segments, plexiform layers, and ganglion cell layer in eyes.

Note: S=subject; E=experimental methods

Table 4. *Summary of NEGATIVE Ngb Expression Studies*

Published year, place, & authors	**Published purpose, subjects, experimental method, time, and tissue**	**Results**
Li, R.C., et. al 2006, USA	S: Male Sprague-Dawley rats (172-225g) E: Hypoxia (SH:10% O_2) or intermittent hypoxia (IH:10% and 21% O_2 alternating every 90' for 1, 3, 7, & 14 days Western blot, RT-PCR	SH: Ngb mRNA and protein (-) IH: slight in Ngb expression at day 1(-)
Schmidt-Kastner, R. et. al 2006, USA, Germany, & Netherlands	S: Rat brain E: Hypoxia (0-1% O_2 for 24 hrs) RT-PCR	Ngb m RNA: (-)

Note: S=subject; E=experimental methods

2.5 NEUROGLOBIN IN THE ISCHEMIC STROKE MODEL

No research currently exists on Ngb in the clinical TBI literature. However, there is some research on Ngb in ischemic stroke. The earliest of this research was published by Greenberg and colleagues in 2001. They developed an experiment wherein they cultured mouse neuronal cells both with and without oxygen for 24 hours *in vitro*, and found that the cells that were deprived of oxygen produced higher levels of Ngb protein and mRNA than the oxygenated cells (Sun et al, 2001). To determine if this finding could be replicated *in vivo*, they occluded the middle cerebral arteries of rats for 90 minutes, and found that the hypoxic state resulted in higher expression of Ngb protein and mRNA in the rat cortical cells (Sun, Jin, Peel, Mao, Xie & Greenberg, 2001). They concluded that Ngb acts as an endogenous neuroprotector for cerebral ischemia. Venis et al (2001) reported that when mouse cells were engineered to either over or under express Ngb, increasing Ngb expression lessened hypoxic injury in the form of less cell membrane disruption, whereas reducing expression worsened the injury. This work implies that Ngb may serve to lessen brain injury due to ischemic stroke. Then in 2003, Sun, Jin, Peel, Mao, Xie, & Greenberg again used an experimental *in vivo* model. They found that cerebral infarct size was increased in rats given Ngb antisense oligodeoxtnucleotide via an intra-cerebrovascular route. They also found that the infarct size was reduced by 56-60% ($p < 0.02$) after intracerebral administration of an adeno-associated virus vector that expressed Ngb after 24 hours. These experiments seem to indicate that Ngb expression is enhanced in acute cerebral hypoxic states, although in these studies Ngb expression was artificially over expressed. However, Mammen, Shelton, Goetsch, Williams, Richardson, Garry and Garry (2002) observed no global changes in Ngb expression in

the brains of mice exposed to chronic hypoxic conditions, implying that Ngb is an acute phase substance and might have implications in the acute treatment of ischemic stroke but perhaps not in chronic states.

Hundahl, C. et al. (2006) examined post-ischemic Ngb expression *in vivo* in 10 spontaneously hypertensive rats who were subjected to MCA occlusion for 90 minutes, (N = 6) or sham (N = 4) and then euthanized 24 hours later. They examined post-ischemic Ngb expression and the neuronal marker NeuN using free-floating immunochemistry. They found that significantly less Ngb mRNA was expressed in the ischemic hemispheres of the MCA occluded animals after 24 hours ($p < 0.002$). At the protein level, they found significantly lower numbers of Ngb and NeuN-positive striatal neurons in the MCA occluded rats ($p < 0.004$). They also found that Ngb expression was mainly confined to the hypothalamus and amygdale. In the ischemic area they did not observe selective sparing of Ngb expressing neurons. Although this study produced negative findings, they hypothesized that the 24-hour time point may have been too late. Nevertheless, they concluded that there is a lack of increased Ngb expression within and adjacent to the infracted region of spontaneously hypertensive rats after MCA occlusion, although the finding might not be replicated in other rat strains.

Nevertheless, Greenberg's group, the most widely published on this topic, prepared a review article (Greenberg, Jin and Khan, 2008) and concluded that Ngb expression is likely induced by neuronal hypoxia and cerebral ischemia, and that Ngb protects neurons subjected to profound hypoxia or focal cerebral ischemia. They recommend further study to determine the mechanisms whereby Ngb is induced by hypoxia, as well as the mechanisms whereby it provides protection. They hypothesize that Ngb induction might be preceded by HIF-dependent and/or independent pathways, and that the mechanisms of protection might include oxygen transport,

RS/NOS scavenger capability, G-nucleotide disassociation inhibition, and also functionality as an O_2 sensor that might trigger downstream adaptations under hypoxic conditions.

2.6 THE GENETICS OF NEUROGLOBIN (NGB)

Ngb is located in chromosome 14. The length of chromosome 14 is 106,368,585 bps, there are 662 known protein-coding genes, 37 novel protein coding genes, 60 pseudogene genes, 62 mRNA genes, 11 rRNA genes, 42 snRNA genes, 61 snoRNA genes, 41 misc RNA, and 392,690 SNPS (http://www.ensembl.org/Homo_sapiens/mapview?chr=14). There are 103 core nucleotides for Ngb (http://www.ncbi.nlm.nih.gov).

Neuroglobin (Ngb) has 5.822 kbp (1,000 base pairs) from chromosome 14, positioned on 76,801,586 to 76,807,407 and four reference genomic sequence SNPs with Ngb's location: rs3783989, rs3783988, rs10133981, and rs7149300 (http://www.hapmap.org). According to the international HapMap project, rs3793988 (SNP1; organism: human; alleles= A/G; genomic location is on chromosome 14, 76804333 to 76804333, negative strand relative to the human reference sequence) was highly correlative with rs1013398, which was chosen in this study. The HapMap used Utah residents with roots in northern and western Europe (CEU), a population similar to that of the study population (90% Caucasians) and Yoruba in Ibadan, Nigeria (YRI): A/A; 0.729, count = 43; A/G, 0.237, count =14; and G/G, 0.034, count = 2) (10% Blacks). This HapMap also identified the reference homozygote genotype frequency as A/A (0.567; count = 34), heterozygote genotype as A/G (0.417; count = 25), and other homozygote genotypes as G/G (0.017; count = 1). Allele frequencies for SNP1 (rs3783988) in CEU are A (0.775, count = 93), G (0.225; count = 27) and in URI area (0.847, count = 100) and G (0.153, count = 18). The

summary of the population diversity shows that the average heterogenic/standard error is 0.419 ± 0.184, individual count = 307, founders count = 247, individual overlap = 58, and genotype conflict = 0. The validation summary is unknown in the marker display's Mendelian segregation, while the PCR results were confirmed in multiple reactions except for the homozygotes detected in individual genotype data. In the SNP1 (rs3783988) diagram below, blue represents the allele present in the human genome assembly as the reference allele, and red represents the alternative allele.

The same results occur for the rs10133981 (SNP2; organism: human; alleles = G/T; genomic location is on chromosome 14, 76805546-76805546, + strand relative to the human reference sequence) with alleles G/T in bdSNP report in the international Hapmap project, CEU had total count 60% with 1) reference-homozygote genotype GG with frequency 0.9 (count = 54), 2) G/T genotype frequencies heterozygote 0.1 (count = 6), and 3) other homozygote T/T frequency 0 (count = 0). Some allele frequencies appear with reference-allele G (0.95, count =114); allele T has 0.05 (count=6) with a total count = 120 in CEU. In the YRI reference-homozygote genotype, G/G is 0.3 (count =18), heterozygote G/T is 0.55 (count=33), and other homozygote genotype T/T is 0.15 (count =9) with a total count that equals 60. Reference allele G is 0.575 (count=69) and other allele T is 0.425 (count= 51) with a total count that equals 120. The summary of population diversity is that the average of heterogenic/standard error is 0.239 ± 0.25, individual count = 270, founders count = 210, individual overlap = 0, and genotype conflict = 0. Validation summary for rs10133981 is still unknown in the marker display's Mendelian segregation, the PCR results confirmed in multiple reactions, and in the homozygotes detected in individual genotype data.

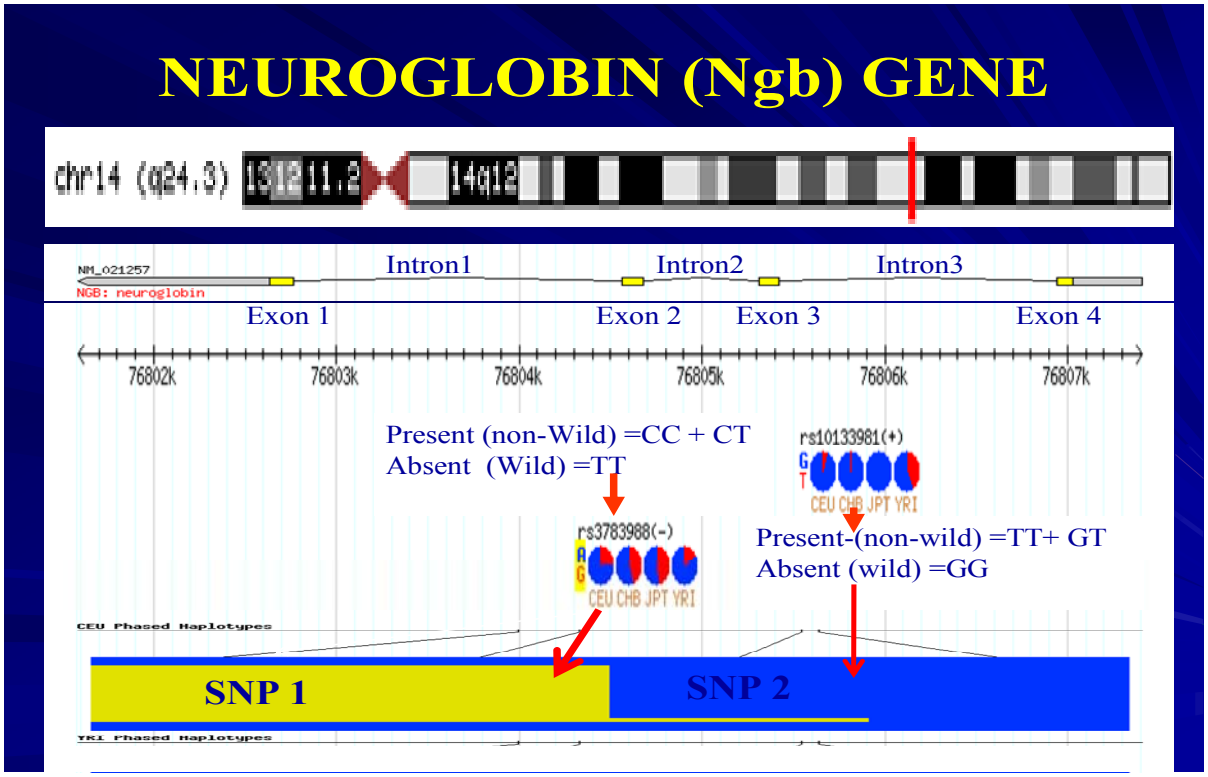


Figure 12. Two Single Nucleotide Polymorphisms of Ngb

2.7 SUMMARY OF LITERAUTRE

Ngb is a novel protein which has been found to be expressed during ischemic conditions in animals, and is thought to have important roles in improving oxygen delivery and utilization. There is little evidence to date examining the expression of Ngb protein or mRNA in humans, warranting further study into whether or not these same protective mechanisms could be identified and utilized to improve outcomes for TBI patients.

2.8 SIGNIFICANCE TO NURSING

Ngb is a novel protein thought to assist in brain oxygen transport and utilization, or as a scavenger of harmful substances, and is expressed during acute hypoxia or ischemia. If present, this protein is hypothesized to not only maintain function in neurons through improved O_2 delivery, but also decrease the damage due to secondary brain injury. Although nurses in the acute care setting cannot impact primary injury sustained at the time of TBI before the patient presents, they can perform functions that either directly limit secondary injury, or promote the patient's own innate physiologic mechanisms for protection. If Ngb can be proven to be present in humans and assist in brain oxygen transport or utilization, or as a scavenger of harmful substances of oxidative stress, then nurses may be able to use this information to determine which patients are more likely to have better outcomes. They might also be able to target which patients are more at risk for secondary injury development requiring a higher level of monitoring. Furthermore, it is possible that they could develop or assist in the development of interventions to enhance Ngb expression.

2.9 INNOVATION

Identification of genetic biomarkers is vital to our understanding of important mechanisms for protein and peptide information through the entire genome sequence. Ngb is a novel protein about which little is known, and even less is known about its development, presence and actions in humans who have experienced TBI. There is variability in the success of TBI recovery that cannot be explained by the extent of primary injury alone. It is possible that Ngb may contribute

to this variation. If Ngb protects the cerebral neurons in hypoxic/ischemic states, it will be an important factor in understanding the variance in TBI recovery, and in possibly developing new therapies to treat patients with, or at risk for, neurological damage due to secondary injury.

3.0 PRELIMINARY STUDIES

This chapter discusses the methodologies and results for three preliminary studies. Parent Studies NIH NR04801 and NS30318 previously collected and stored all samples utilized in the preliminary studies and intended for use in this dissertation study. The two parent studies include the effect of ApoE on outcomes in traumatic brain injured adults, and the effect of inherited factors (genes) on the brain's response during the first five days after TBI. These parent studies also provided the samples utilized in the 3 preliminary studies described below.

3.1 PRELIMINARY STUDY # 1

Chuang, P.Y., Alexander, S., & Kim, Y. The relationship between cerebral spinal fluid lactate/pyruvate and cerebral blood flow following traumatic brain injury. ENRS 18th Annual Scientific Sessions "New Momentum for Nursing Research: Multidisciplinary Alliances" April 20-22, 2006.

The first preliminary study focused on the relationship between cerebral spinal fluid lactate and pyruvate on cerebral blood flow during the first five days of TBI patient admission. **Purpose**: The objective of this study was to examine the relationship between CBF and CSF lactate/pyruvate (L/P) ratios. **Methods**: The sample consisted of sixty-four patients (19 females, 45 males) with severe TBI who were admitted to the Level 1 Trauma Center at the University of

Pittsburgh Medical Center (UPMC) from 1995-2000. The Institute Review Board (IRB) of the Traumatic Brain Research Center (TBRC) approved all subjects. Inclusion criteria were: 1) age 16 to 75 years; 2) GCS $\leq$ 8; 3) positive CT findings for TBI; 4) CSF lactate and pyruvate data available; and 5) Xenon cerebral blood flow data available. Exclusion criteria were: 1) cardiac arrest; 2) respiratory arrest; and 3) meeting brain death criteria. The Brain Trauma Foundation's Guidelines for the Management of Severe Head Injury determined the course of treatment including placement of an EVD catheter, central line, and arterial line. As per this protocol for standard of care, the ICP was maintained $\leq$ 20 mmHg, and the CPP maintained $\leq$ 60 mmHg. CSF was collected from the EVD every four hours during the first 24 hours and every six hours during the next four days for lactate and pyruvate measurements. Xenon Computed Tomography (Xenon CT) utilizes CT technology to identify uptake of Xenon gas into cerebral tissue. The Xenon CT CBF data is reported as average CBF for the entire brain (global) measured on day 1, day 3, or day 5 after TBI. The CSF lactate and pyruvate levels taken closest in time to the Xenon CT scan were used for this analysis. The mean and range were computed for CSF lactate, pyruvate, lactate/pyruvate ratio, and Xenon CBF for the entire sample. Mean Xenon CBF was obtained by averaging the mean left and right hemispheric CBF across 4 levels. Low CBF was defined as mean CBF $\leq$ 40 ml/g/min, and normal CBF as > 40 ml/g/min. Descriptive and correlational analyses were used to examine the relationship between CSF L/P ratio and CBF. **Results**: The sample had a mean age of 30.5 years (SD = 14) and was primarily male (73%) and Caucasian (87.5%). Mean GCS was 5.7 (SD = 1.4, Mode = 7). CSF L/P ratio was drawn an average of 1.5 (SD = 7.0) hours before XeCT. Thirty one subjects (48.4%) exhibited low CBF. No significant relationship was shown between CSF L/P ratio and low CBF (p = 0.13). Low CBF was significantly associated with race (p = 0.02) with more black subjects (87.5%)

developing low CBF than Caucasian (42.8%). **Conclusions**: There was no relationship between the CSF L/P ratio and a low CBF following TBI. The significant correlation between race and low CBF was of interest and warrants additional research with a larger sample. There might be a reason for the lack of a relationship between the L/P ratio and the low CBF. The testing time of each subject for the lactate, pyruvate, and L/P ratio differed and Xenon CT, it was hard to match all variables (lactate, pyruvate, and L/P ratio and Xenon CT) together. There might be another as yet unidentified issue that caused the results to not be significant.

Skills learned in this Preliminary Study #1 which will contribute to the proposed study: a) increase understanding of how biochemical pathways may contribute to secondary injury in TBI patients, b) increase understanding of HPLC methodology to detect biomarkers, c) began acquisition of skills to access and utilize the databases from the parent study, d) performed statistical analyses using parent study data.

3.2 PRELIMINARY STUDY # 2

Chuang, P. Y[1].; Alexander, S[1].; Poloyac, S[2].; Hravnak, M[1]. The Role of Neuroglobin in Response to Cerebral Hypoxia/Ischemia after Traumatic Brain Injury. AANN, 2008.

Purpose: To determine if neuroglobin (Ngb), a novel oxygen transporter protein, is detected in fresh (immediately drawn) or bagged (aggregate 12 hr) CSF samples collected 1-5days after severe TBI using Western Blot methodology. **Background/Significance:** Cerebral ischemia causes secondary brain injury following TBI. In animal models, Ngb is hypothesized to increase the availability and delivery of oxygen to neuronal tissues during cerebral hypoxia, and has been detected by Western Blot testing. Only one study has identified Ngb in human CSF, using mass

spectrometry in females with chronic pain. No study has yet detected the presence of Ngb in human CSF during cerebral ischemia by any method. **Methods:** CSF was collected from 17 subjects with severe TBI (days 1-5) who were enrolled in two larger studies (NIH NR008424 and NS30318) from 2003-2006 who met the following criteria: age 16-75 yrs; Glasgow Coma Scale (GCS) $\leq$ 8, positive computerized tomography scan, external cerebral ventricular drainage device present, and no cardiac or respiratory arrest before admission. The Lowry Assay and Western Blot were used for protein analysis with protein extracts (5-25 μg per lane) in 12-15% SDS polyacrylamide gel. We used mouse monoclonal antibody as the primary anti-human Ngb antibody and goat anti-mouse IgG_1 as the secondary antibody to adhere human Ngb, if present, to the chemiluminescence membrane for visualization. **Results:** Thirty CSF samples were available (14 fresh; 16 bagged) for analysis taken an average of 51.6 hours after TBI. Patients were primarily young (mean age 33.7, range 18-57 yrs.), female (53%), and Caucasian (90%), with a mean GCS of 5.43 (SD ± 1.59). When chemiluminescence, the most sensitive analytic segment of the Western Blot test, was performed to evaluate for the presenceand quantification of protein staining, no human Ngb was detected in any fresh or bagged CSF samples. **Conclusion:** Western Blot, a readily available testing method, was not able to detect Ngb in the CSF of patients with severe TBI, whether the sample was freshly drawn from the ventriculostomy or in an aggregate sample allow sitting at room temperature for 12 hours. While Ngb expression is associated with cerebral ischemia in animal models, further research is needed to determine if Ngb is present in humans with severe TBI using detection methods other than Western Blot. Such information will help to develop mechanisms to limit secondary injury.

Skills learned in this Preliminary Study #2 which will contribute to the proposed study: a) independence in performing Western Blot protein analysis method, b) laboratory skills in handling and storing human CSF specimens, c) development of technical skills in the laboratory such as pipetting and centrifuging, d) performing laboratory skills repeatedly to obtain a results that are not confounded by inconsistency in technique, e) self protection using blood and body fluid precautions.

3.3 PRELIMINARY STUDY # 3

The third descriptive study was designed to examine Ngb expression in the brains of two non-ischemic and one ischemic adult Sprague-Dawley male rat. **Purpose:** The purpose of this study was to determine if Ngb could be isolated from ischemic brain tissue in an animal model of stroke. **Methods:** The subjects (300-350 g) were divided into the following groups: non-ischemic (sham control; N = 1) or global cerebral ischemia (N = 2) in which the MCA was occluded (MCAo) for 90 min, followed by 30 hr of reperfusion. Western blots were used to analyze Ngb protein presence in the brain. The Institutional Animal Care and Use Committee reviewed and approved all procedures involving the animals. Anesthesia was induced by the inhalation of 5% isoflurane delivered in a mixture of nitrous oxide and oxygen (70%/30%) at 1.0 L/min for 5 min. The MCAo produced an ipsilateral infarct that affected specific brain regions. Completeness of the lesion was subsequently verified behaviorally by a surgeon who examined the postoperative movements of each rat's extremities. Changes in blood flow are not likely to explain the protective effect of Ngb expression following local cerebral ischemia. Animals that were randomly assigned to receive sham surgery received the same operative procedures except

that the middle cerebral artery was not occluded. After MCAo, the animals were returned to their units in the laboratory of Bioscience III for 90 min until the filament was removal from the artery (onset of reperfusion). After 30 hr, all of the rats were sacrificed. Brain tissues were analyzed by Western blotting for Ngb presence. Brain tissue samples were removed from the rats and the fresh tissues were analyzed immediately. Other frozen brain tissues were stored until the Western blots were performed. All frozen brain tissues were unfrozen and homogenized in lysis buffer that contained a protease inhibitor cocktail (1:1) (Sigma cat # MFCD00677817), incubated on ice for 5 min, and swirled over forty times at 4° C. The brain lysates were transferred to an eppendorf tube and centrifuged at 12,000 rpm for 30 min. A Lowry protein assay was performed in order to calculate the protein concentration of the brain tissues. Subsequently, Western blot analyses were performed to assess for Ngb presence. **Results:** Western blotting did not reveal Ngb presence in the non-ischemic or ischemic brains of Sprague-Dawley male rats.

Skills learned in this Preliminary Study #3 which will contribute to the proposed study: a) development of technical skills in the laboratory such as pipetting and centrifuging, b) performing laboratory skills repeatedly to obtain results that were not confounded by inconsistencies in the techniques, and c) acquisition of skills for performing tissue lysis.

3.4 SUMMARY OF PRELIMINARY STUDIES

Disappointingly, Ngb was not detected using Western blot techniques in the CSF of humans with ischemic brain injuries or in the ischemic brains of rats (see Figure 13). This finding does not necessarily mean that Ngb is not expressed, but rather that Western blotting may not be the best detection method. Possibly, detection would be improved by using mass spectrometry, a

quantitative sample analysis technology used to measure the mass-to-charge ratio of ions transported by magnetic or electric fields. However, mass spectrometry analyses are extremely expensive to perform. Before mass spectrometry analyses are used to assess Ngb in humans, a reasonable first step is to determine if humans differ in their genetic predisposition to developing the protein. If a genetic predisposition can be identified, which is more cost effective to assess, such information would provide justification for the expenses associated with using mass spectrometry to identify Ngb.

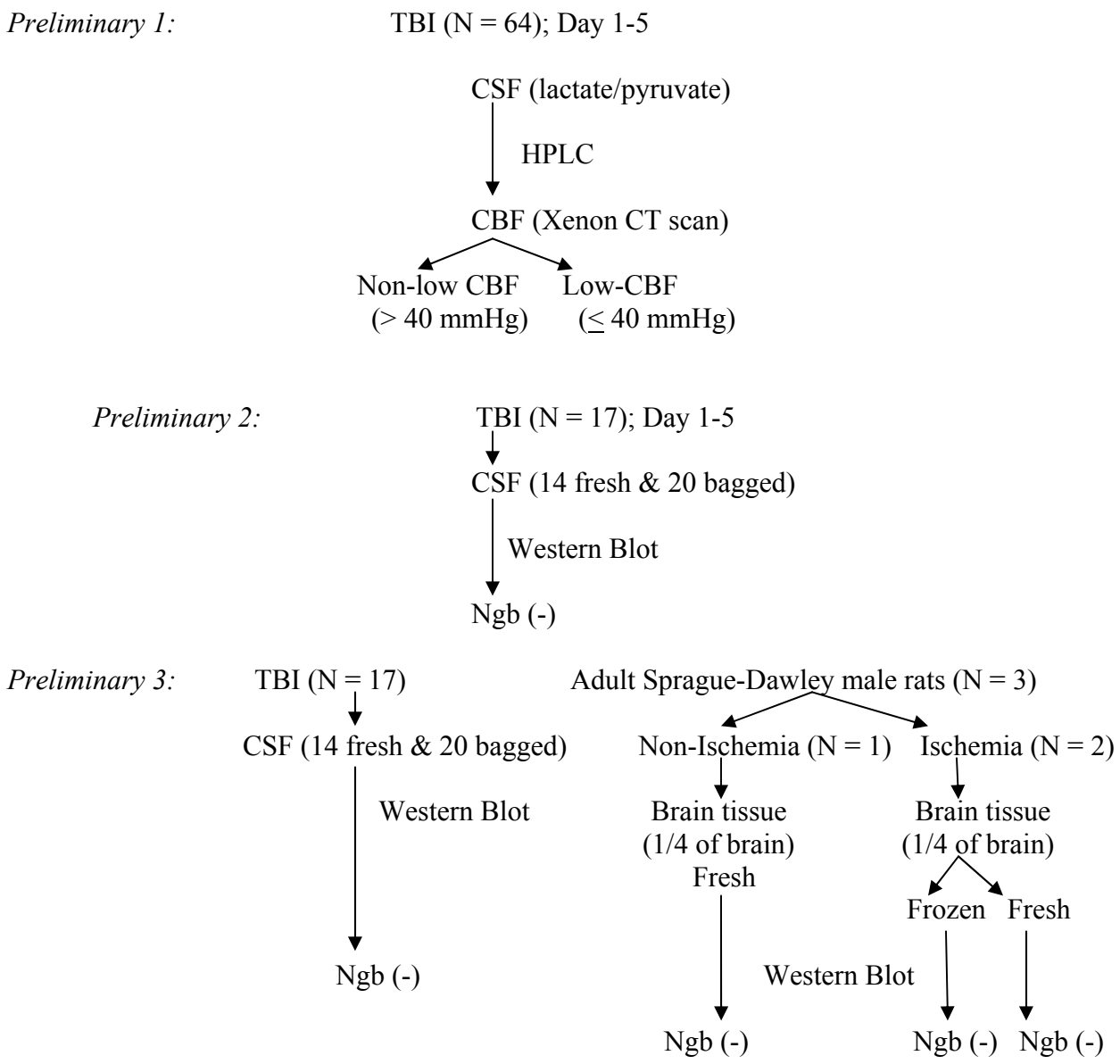


Figure 13. Summary of Preliminary Studies

4.0 METHODS

This chapter details the methods used to conduct the proposed research study, including design, setting, samples, the methods used to determine the Ngb genetic variants, justification for the sample size, instruments (GCS, GOS, and Xenon CT), procedures, and data analyses.

4.1 RESEARCH DESIGN

This study has a prospective, descriptive, comparative design that utilizes DNA samples extracted from the blood and CSF of the 216 patients obtained for the two parents in which the recovery of the patients was followed for the first five days following severe TBI. The genetic testing was conducted by the doctoral candidate. The data collected at 3, 6, 12, and 24 months by a neuropsychology technician regarding mortality and the scores on the GOS, the Disability Rating Scale (DRS), and the Neurobehavioral Rating Scale (NRS) were available from the parent study for a subset of this population. The demographic outcome variables of the present experiment were limited by the information collected in the two parent studies.

4.2 RESEARCH SETTING

The parent studies were conducted at the UPMC, which has four neurotrauma intensive care units. These units provided care for the 216 patients with TBI in this study. Stored CSF and blood samples were accessed from the Brain Trauma Research Center (BTRC) at the UPMC. All patients with TBI were admitted and enrolled in the parent BTRC study after being diagnosed with severe TBI between 2000 and 2006. The laboratory analyses for the current study were performed in the molecular genetics laboratory of Dr. Yvette Conley, School of Nursing, University of Pittsburgh.

4.3 RESEARCH SAMPLE

All patients admitted with a severe TBI between January 2000 and December 2006 were screened and enrolled in the BTRC parent studies. Inclusion criteria for the BTRC studies were as follows: 1) GCS $\leq$ 8 without paralytics or sedatives, 2) head CT scan positive for closed head injury, 3) aged 16-75 years, and 4) EVD available. Exclusion criteria for the BTRC were: 1) mental retardation or cognitive deficit before injury, 2) cardiovascular arrest, 3) respiratory arrest, and 4) met brain death criteria.

Children under 16 years of age were not included in this study because there is evidence to suggest that children recover from TBI differently than adults. Patients with baseline mental retardation or other cognitive deficits before the injury were excluded to avoid confounding the analysis of the outcome measures. We anticipated that this sample would be 90% Caucasian and

75% male, consistent with the TBI population presenting to the UPMC Presbyterian hospital. No inclusion or exclusion criteria were based on race or gender.

4.4 SAMPLE JUSTIFICATION

Generally, larger sample sizes decrease the variability within the sample means in quantitative research studies. Conducting such a study depends on a variety of factors, such as the null hypothesis (one or two-tailed), power (1-β), significance (α), and sample size (N). However, the two parent studies from which the sample for this study was drawn were limited to 216 patients available from the years 2000 to 2006. Analysis of the parent sample was based on the frequencies of the SNPs, which varied with race. The final data analyses were performed on 208 patients who all met the inclusion criteria; detailed information is included in Chapter 5. The racial identities of the patients included 196 (77.9%) Caucasians, 10 (4.8%) Blacks, and 2 (1.0%) American Indian, or Alaskan Native. Therefore, based upon the HapMap's analysis report (http://www.HapMap.org, 2008), the chosen SNP sequences for Ngb were rs3783988 (SNP1), which represents 57% A/A nucleotide pairs, 41% A/G nucleotide pairs, and 2% G/G nucleotide pairs at the polymorphic site in U.S. residents with northern and western European ancestry according to the Centre d'Etude du Polymorphisms Human (CEPH or CEU), and rs10133981 (SNP2), which represents 90% G/G pairs, 10%G/T pairs, and 0% T/T pairs at the polymorphic site according to the CEU. Thus, the frequencies of the SNPs determined the available sample. PASS and NCSS statistical software packages were used to examine sample power.

The sample size of 208 participants was powerful enough (100%) to detect a difference of -1.0 between a hypothesis stating no between-group mean differences and a hypothesis stating

between-group mean differences of 1.0, with an estimated standard deviation of 0.0 and a significance level (alpha) of 0.05 using a two-sided one-sample t-test. In a one-factor ANOVA power analysis, estimated sample sizes were provided by the software of 108, 86, and 21 for the three groups means that were to be compared. The total sample of 215 patients achieved 100% power using an F test with a 0.05 significance level. The size of the variation in the means is represented by the standard deviation and was 0.66. Individual data points were considered outliers if they were larger than one standard deviation from the group mean (Desu & Raghavarao, 1990; Fleiss, 1965; Kirk, 1982).

4.5 RESEARCH SETTING HUMAN RIGHTS PROTECTION

The Institutional Review Board of the University of Pittsburgh (see Appendix) approved the parent studies, and approval for the current study was covered under the original consent. Informed consent was obtained from all participants in the parent studies. Confidentiality of the patients was maintained in the parent studies by assigning a unique identification code to each patient. All data and samples were labeled with the unique identification code. A file linking the unique identification code and the patient identifiers was maintained in a separate, locked filing cabinet within the BTRC. All samples and data used for the purpose of the proposed study were retrieved in a de-identified state. The confidentiality of the patients was maintained by keeping identifying data on a password-protected computer that was accessible only to the Principle Investigator of the parent study. No identifiable information was available in the databases from which data were extracted for the purposes of this current analysis.

4.6 RESEARCH PROCEDURES-PARENT STUDIES

4.6.1 Patient care in the parent study

Patients in the parent studies underwent an admission protocol according to a standardized physician order set (Appendix A). The order set included protocols for admission, assessments of intracranial hypertension, and maintenance of ICP, sedation, and fluid resuscitation for patients with severe TBI after admission to the UPMC. The Admission Protocol included directives for vital signs, patient care (routine neurologic management of patient positioning and EVD), intravascular fluid, medications, laboratory tests, research procedures after consent, and radiology.

The Intracranial Hypertension Protocol was a second set of standardized orders that were in place to maintain the ICP $\leq$ 20 mmHg. The protocol provided directives for 1) ICP > 20 mmHg, 2) ICP consistently $\geq$ 25 mmHg after step 1, and 3) ICP consistently $\geq$ 25mmHg after step 2.

The ICP Maintenance Protocol was a third set of standardized orders that described the goals, triggers, interventions, and physician notifications. The main goals of this protocol were to provide a standardized approach to ICP management in patients with severe TBI and to provide the nurses with guidance in the next level of control over elevated ICP.

The Sedation Protocol was a fourth set of standardized orders that included assessments and interventions for sedation protocol, analgesia (Fentanyl), and neuromuscular blockade (Vecuronium).

The fifth set of standardized orders was the Fluid Resuscitation Protocol which described physician notifications, fluid challenge, and vasopressor administration. Table 5 outlines the order of steps to be implemented if hypotension or low cerebral perfusion pressures occurred.

Table 5. Hypotension Protocol in Parent Studies (MAP < 65 mmHg)

check	CVP	Challenge Volume	Fluid	Frequency	Maximum Volume
	< 5	1000 ml	Colloid	Bolus	1000 ml
			Sodium Chloride	q 30 min	2000 ml
	5-10	500 ml	Colloid	q 15 min	1000 ml
			Sodium Chloride	q 15 min	1000 ml
	11-12	250 ml	Colloid	q 10 min	500 ml
			Sodium Chloride	q 10 min	500 ml
Low Cerebral Perfusion Pressure (CPP < 60 mmHg)					
	CVP	**Challenge Volume**	**Fluid**	**Frequency**	**Maximum Volume**
	< 5	1000 ml	Sodium Chloride	q 60 min	2000 ml
	5-10	500 ml	Sodium Chloride	q 60 min	1000 ml
	11-12	No fluid challenge, norepinephrine (Levophed) if MAP < 100 mmHg			

Key: MAP = mean arterial pressure, CVP = central venous pressure

4.6.2 CSF and blood sample collection and storage-parent studies

The bagged CSF samples from the parent study were collected by passive drainage from the EVD into a CSF collection bag at room temperature, located at the patient's bedside. The drained CSF was collected every 12 hr by changing the CSF collection bag. CSF (3-5 ml) was collected using sterile techniques as per hospital policy. Samples were aliquoted into cryogenic tubes and initially stored in a refrigerator located directly outside the ICU. On the fifth day post TBI, when sample collection was completed, all CSF specimens were moved to a -80º C freezer located in the School of Nursing for long term storage.

At the 12-hour collection time point, a blood sample was also obtained. Specifically, 3 ml of blood was obtained from the patient's indwelling arterial line or central venous catheter and placed into a laboratory tube. The sample was placed in the refrigerator located directly outside of the ICU and subsequently transferred to Dr. Conley's laboratory at the School of Nursing and processed within 48 hr.

4.6.3 CT scan-parent studies

All patients underwent a CT scan, which creates cross-sectional images of the structures in the body. It is a non-invasive procedure; x-rays are taken from many different angles and processed through a computer to produce a three-dimensional image called a tomogram. CT is used to detect abnormalities such as blood clots, cysts, fractures, infections, and tumors in internal structures (e.g., bones, muscles, organs, soft tissue). The procedure also may be used to guide the placement of instruments within the body (e.g., to perform a biopsy). Contrast agent (e.g., iodine-based dye, barium solution) may be administered orally or injected into a vein prior to the CT scan to allow the organs and structures to be seen more easily. Patients usually are instructed not to eat or drink for a few hours prior to administration of the contrast agent because the dye may cause stomach upset. There are no side effects of the procedure except for possible reactions to the dye (rash, itching or feelings of warmth in the body). Patients consented at the time of the parent studies.

4.6.4 CT scan-parent studies clinical assessment and outcome instruments (GCS, GOS, DRS, and NRS)-parent studies

The purpose of this section is to describe the clinical assessment tools used in the parent study, namely the GCS, which was used to determine TBI severity, and the GOS, which was the primary outcome measure. Two additional measures of outcome, the DRS and the NRS, are also described.

4.6.4.1 Glasgow Coma Scale (GCS) The GCS is the most common neurological scale used in intensive care units to obtain a reliable assessment of the conscious state of a person with a head injury. The scale was published in 1974 by Graham Teasdale and Bryan J. Jennett, professors of neurosurgery at the University of Glasgow. The test (see Table 6) measures eye opening, verbal response, and motor response. The final score is obtained by adding the subscores from the three domains, with a possible total score of 15. The lowest score on the GCS is 3 and indicates deep coma or death. Severe injury is indicated by GCS $\leq$ 8, moderate injury is defined as GCS = 9-12, and a minor injury is defined as GCS $\geq$ 13. In some special cases, patients cannot respond to the GCS parameters; therefore, clinicians can record markers for conditions of paralysis , incubated or tracheotomized, untestable, sedation, and eyes swollen or closed (Fisher & Mathieson, 2001).

Table 6. Glasgow Coma Scale (GCS)

Eye Opening (E)	Verbal Response (V)	Motor Response (M)
4=Spontaneous	5=Normal conversation	6=Normal
3=To voice	4=Disoriented conversation	5=Localizes to pain
2=To pain	3=Words, but not coherent	4=Withdraws to pain
1=None	2=No words, only sounds	3=Decorticate posture
	1=None	2=Decelerate
		1=None
		Total = E+V+M

Score is 13-15: mild injury.
Score is 9-12: moderate injury and disability which included loss of consciousness for greater than 30 minutes and physical or cognitive impairments which may or may resolve with rehabilitation.
Score is 3-8: severe injury or coma in an unconscious state. No meaningful response, no voluntary activities.
Score is 3: Vegetative State, sleep-wake cycles, arousal, but no interaction with environment, no localized response to pain.
Persistent Vegetative State: Vegetative state lasts longer than one month.
Brain Death: No brain function; specific criteria are needed to make this diagnosis.

Gill et al. (2004) noted that the reliability of assessments among evaluators was 32% for total GCS (π-b = 0.739, Spearman p = 0.864, Spearman p^2 = 75%), 74% for components of eye movement (π-b = 0.715, Spearman p = 0.757, Spearman p^2 = 57%), 55% for verbal responses (π-b = 0.587, Spearman p = 0.665, Spearman p^2 = 44%), and 72% for motor responses (π-b = 0.742, Spearman p = 0.808, Spearman p^2 = 65%). In contrast, the disagreement rate for GCS was from 0-0.5 and the low range was 0.0-0.5 and the higher range is 0.3-0.5 (Teasdale et al., 1978; Rowley & Fielding, 1991; Juarez & Lyon, 1995). Teasdale et al. (1978) assessed the disagreement rate of seven neurosurgeons and found low disagreement for 121 ICU patients with GCS scores of 0.005-0.163 for eye opening, 0.000-0.035 for verbal responses, and 0.004-0.064 for motor responses. Rowley and Fielding (1991) noted that the disagreement rate was 0.005-0.163 for assessments of eye opening, 0.000-0.035 for assessments of verbal responses, and 0.004-0.064 for assessments of motor function. Juarez and Lyon (1995) videotaped 7 ICU

patients and found that the disagreement rate for the eyes was 0.00-0.29, verbal was 0.00-0.06, and motor was 0.00-0.22. These research findings revealed that the GCS is a good neurological assessment tool for use with head injury patients.

Although the GCS has been used for over two decades, Waterhouse (2005) recommended that pupil size and vital signs are also important for neurological assessments. Pupil size serves as a window to the brain through the pupillary reactions to light, their shape, size, and symmetry; pupillary changes can indicate subsequent elevations in ICP. Pupillary changes can also directly or indirectly indicate the brain damaged region in early and late stages of recovery. Vital signs can be used to monitor cerebral hypertension and classify irregularities in breathing pattern.

Some investigators examined the possibility that the motor score was the most sensitive and specific portion of the GCS for predicting outcome. Ross and associates (1998) predicted head injury outcome in patients by comparing the motor score component of the GCS to the total GCS score. The results indicated that the sensitivity of the motor score was 91% and the specificity was 85%; the sensitivity of the total GCS score was 92% and the specificity was 85%, as assessed with Receiver Operating Characteristic (ROC) curves (McNett, 2007). Healey and associates (2003) also demonstrated the sensitivity of the motor scores (ROC = 0.87) and the total GCS scores (ROC = 0.89) of 200,000 general trauma patients. Meredith and associates (1995) showed that the motor component of the GCS had 59% sensitivity and 97% specificity rates for predicting outcome.

4.6.4.2 Glasgow Outcome Scale (GOS) The GOS has five categories (dead, vegetative, severely disabled, moderately disabled, and good recovery) and emphasizes physical problems rather than cognitive or emotional problems after head injury (Anderson et al., 1993).

Table 7. Glasgow Outcome Scale (GOS)

State	Definition	Score
Dead		1
Vegetative	No evidence of meaningful responsiveness	2
Severe Disability	Conscious but needs the assistance of another person for some activities of daily living	3
Moderate Disability	Independent but disabled	4
Good Recovery	Capacity to resume normal occupational and social activities	5

Jennett and Bond (1975), who designed the GOS, believe that mental status change is more important than physical limitation in determining disability after head injury. However, good recovery may be defined as physical independence in the absence of neurological deficits (Hutter & Gilsbach, 1993). The GOS takes 5-15 minutes to rate. Interrater reliability among the structured interviews for the five points on the GOS resulted in a weighted kappa value of 0.89, which suggests that patient assessments with the GOS are practical and reliable when a standard format and written protocol are used (Wilson, Pettigrew, and Teasdale, 1998). King, Carlier, and Marion (2005) used the GOS to predict the severity of TBI in patients (GCS $\leq$ 8) at 3 and 12 months following injury. Their results showed that the adjusted logistic model was characterized by a steep gradient for long-term recovery potential that depended upon the GOS score at 3 months, ranging from an 89.4% chance of poor outcome for patients with a GOS of 2 to a 0.11% chance of poor outcome for those patients with a GOS = 5. The 3-month GOS score is a powerful, independent predictor of long-term outcome for patients with severe TBI.

In this study, the GOS was dichotomized into Good Outcome (GOS 4-5) and Poor Outcome (GOS 1-3). This dichotomization scheme was supported by a study from Broessner et al. (2007) who subdivided the GOS in terms of favorable outcome (GOS 4-5) and unfavorable outcome

(GOS 1-3) and analyzed survival, mortality, and long-term functional disability, and determined the long-term outcome for 662 neurologically critical patients in the 2.5 years following the injury. Sakr et al. (2004) also subdivided the GOS into good outcomes (GOS 4-5) and poor outcomes (GOS 1-3) and related electrocardiogram changes to the neurological outcomes of patients with an aneurysmal subarachnoid hemorrhage.

4.6.4.3 Disability Rating Scale (DRS) The DRS (Appendix B) was developed and tested with older juveniles and adults with moderate and severe TBI in a post-trauma setting (Wright, 2000). All three World Health Organization categories were applied: impairment, disability, and handicap (WHO, 2001). The following four domains resulted: a) awareness and responsiveness (eye opening), communication ability, and motor response; b) cognitive ability for self-care activities (feeding, toileting, and grooming; c) dependence on others (level of functioning); and d) psychosocial adaptability (employability) (Wright, 2000). Part A of the DRS is similar to the GCS scale, part B of the DRS reflects the level of disability, part C of the DRS measures the level of function, and part D of the DRS measures employability. The maximum score a patient can obtain on the DRS is 29 (extreme vegetative state); a patient without disability would score zero (Wright, 2000). The levels of disability as assessed with the total DRS score (Wright, 2000) are as follows: 0 indicates no disability, 1 indicates mild disability, 2-3 indicates partial disability, 4-6 is for moderate disability, 7-11 is for moderately severe disability, 12-16 is for severe disability, 17-21 is for extremely severe disability, 22-24 indicates a vegetative state, and 25-29 indicates an extreme vegetative state. Patient assessment with the DRS takes only 30 s to 15 min by trained staff. The reliability and validity of the DRS has been proven by Rappaport et al. (1989), who studied the amount of time that elapsed between injury and recovery by using the

DRS with 63 patients with TBI. The results showed that a significantly greater improvement was seen in the early admission group. In comparing the relative sensitivity of the DRS with the GOS, 71% of individuals with TBI showed improvement on the DRS but only 33% showed improvement on the GOS (Hall et al., 1985). The limitation of the DRS is its relative insensitivity at the low end of the scale (mild TBI) and its inability to reflect more subtle disability.

4.6.4.4 Neurobehavioral Rating Scale (NRS) The NRS (Appendix C) employs the 29 items of the Likert scale and measures cognition and behavioral parameters of brain disease by a rapid bedside assessment of closed head injury and stroke patients and is highly predictive of long-term outcomes (Hilton, Sisson, and Freeman, 1990). The 5 factors (executive/cognition, positive/negative symptoms, mood/affect, and oral/motor) of the revised NRS (NRS-R) revealed acceptable internal consistency (R^2 = 0.62 to 0.88) and a low to moderate interfactor correlation (R^2 = 0.19 to 0.61) and discriminated well between the GOS-defined groups for 210 randomly-chosen closed head injury patients from 11 large regional North American trauma centers for which the primary outcome measurements (GOS) and secondary outcome measurements (DRS and NRS-R) were available (McCauley et al., 2001).

4.7 SUMMARY OF DATA COLLECTION TIMETABLE-PARENT STUDIES

Table 8 describes the timetable for collection of the clinical data for the parent study and the numbers of patients included in the secondary analysis.

Table 8. Data Collection Schema for Outcome Evaluation (N = 208)

Tools	3 months	6 months	12 months	24 months
GOS	167 (80.3%)	161 (77.4%)	141 (67.8%)	104 (50.0%)
NRS	164 (78.9%)	160 (76.9%)	138 (66.4%)	105 (50.5%)
DRS	62 (29.8%)	76 (36.5%)	71 (34.1%)	38 (18.3%)

Key: GOS = Glasgow Outcome Scale, NRS = Neurobehavioral Rating Scale, DRS = Disability Rating Scale.

4.8 RESEARCH METHODS FOR THE CURRENT STUDY

The three main development phases for the TaqMan SNP genotyping assay included: 1) In order to get DNA amplification, the DNA was extracted by the standard operating procedure (SOP) for blood and by the QiAamp DNA midi/maxi procedure for CSF; 2) Allelic discrimination plating was performed and read; and 3) An allelic discrimination assay was analyzed. The DNA extraction technique does not differentiate between those patients who had a blood sample available and those patients who had a CSF sample available. The Applied Biosystems (AB) Company chose the TaqMan SNP because it provides a high probability of detecting polymorphisms present within any human genome; the technique also uses probe and primer chemistry and designs, which includes screening, association, candidate region, candidate gene, or fine-mapping studies. Moreover, the essential SNP probes have 13 bases, which improves mismatched discrimination and are useful for difficult or variable sequences. The Minor Groove

Binder Technology on the 3' end delivers superior allelic discrimination. Detection is achieved through proven 5' nuclease chemistry by means of exonuclease cleavage of a 5' allele-specific permanent dye label signal. A nonfluorescent quencher eliminates background fluorescence and increases sensitivity for the signal (AB, 2007).

4.8.1 DNA extraction

The DNA extraction procedure for blood used the SOP and the QiAamp DNA midi/maxi protocol for CSF. The purpose of the SOP and the QiAamp DNA midi/maxi was to get large-scale genomic DNA amplification.

4.8.1.1 DNA extraction from blood The SOP for DNA extraction consisted of two steps: blood processing and DNA extraction. Each SOP procedure allowed 16 samples to be processed at one time. The blood processing had five steps. First, the sample specimens were checked for the TBI study name, type of specimen, date, and time. Second, the blood samples were removed from the -20°C freezer, placed in spin tubes, and centrifuged at 2500 rpm for 20 min at room temperature (22-25° C). While the tubes were spinning, the lysis solution, which consisted of 40 ml of ammonium chloride and 5 ml of ammonium bicarbonate, was prepared and maintained at 4º C. Third, 3 ml lysis solution was added to a labeled 50-ml conical tube and supplemented with 200 ul of 10% SDS and 500 ul of freshly-made proteinase K solution (10% SDS, 0.5 M EDTA, 18 mg proteinase K, 8.1 ml sterile H2O). Fourth, the tubes were sealed with parafilm, placed in a 37 °C oven, and rotated over night. The tubes were subsequently removed from the oven and 1 ml of 6 M (saturated) NaCl was added to each tube, which was then shaken for about 15 s until foamy, and then centrifuged for 15 min at 2500 rpm. After centrifugation, the lysis solution in

the sample (supernatant) was decanted, without losing the plasma and pellet, which are then carefully transferred to a labeled 15-ml conical tube with two times the volume of 70% alcohol until the DNA appeared white and stringy and microfuged for 10 min. The tubes were then placed in a 37° C oven until the ethanol had evaporated. Subsequently, the DNA was resuspended in 1 Ml TE buffer. The extracted DNA was ready for amplification. (See SOP for DNA Extraction in Appendix D).

4.8.1.2 DNA extraction from CSF The QiAamp DNA midi kit provides the fastest and easiest way to purify total DNA from CSF. The total genomic or mitochondrial DNA can be purified from 0.3-2 ml or 3-10 ml, respectively. With a suitable centrifuge rotor, eight samples can be prepared simultaneously in approximately 1.5 hr, with approximately 30 mins of hands-on time (QIAGEN, 2008, http://www.qiagen.com). The purified DNA is virtually free of protein, nucleases, and other contaminants or inhibitors of downstream applications. The QiAamp DNA extraction process was similar to the SOP for blood. First, 200 ul of protease, 2 ml CSF, and 2.4 ml Buffer AL were mixed together in a 15-ml conical tube at 70° C for 10 min. Then, 2 ml of 96-100% ethanol was added, and the samples were vortexed. Half of each solution was transferred into a 15-ml conical tube and centrifuged at 3000 rpm for 3 min. Buffer AW1 (5 ml) was added to a QiaAmp midi column and centrifuged at 5000 rpm for 1 min. Buffer AW2 (2 ml) was added to the column and centrifuged at 5000 rpm for 15 min; the column was then inserted into a clean 15-ml conical tube. Buffer AE (300 µl) was added to the column, incubated at room temperature for 5 min, and then centrifuged at 5000 rpm for 5 min more. Another 300 ul of Buffer AE was added to the column, incubated, and centrifuged for 5 min. The DNA (1.5 µl) was now ready for DNA amplification. The protocol for the QiAamp DNA midi kit is included as Appendix B).

4.8.2 TaqMan PCR genotyping assay

The TaqMan PCR assay (called the 5' nuclease allelic discrimination assay) is used to genotype SNPs using a simple technique (Livak et al., 1995). TaqMan PCR involves exponential amplification of almost any region of a selected DNA molecule and is similar to DNA replication in nature (http://www.ncbi.nlm.nih.gov/). In this method, the region flanking the polymorphism (average of about 100-150 bp) is amplified in the presence of two probes, each specific for one allele. Probes are labeled with fluor, called the reporter, at the 5' end, which fluoresces when free in solution, and labeled with a quencher at the 3' end that absorbs the fluorescence from the reporter. During PCR, the Taq DNA polymerase encounters a probe specifically base-paired with its target and unwinds it. The polymerase cleaves the partially unwound probe and liberates the reporter fluor from the quencher, increasing net fluorescence. Fluorescence is a measure of the amount of binding of each probe to a specific allele. The presence of two probes, each labeled with a different fluor, allows one to detect both alleles in a single tube (Hui, DelMonte, and Ranade, 2008) (see Figure 14).

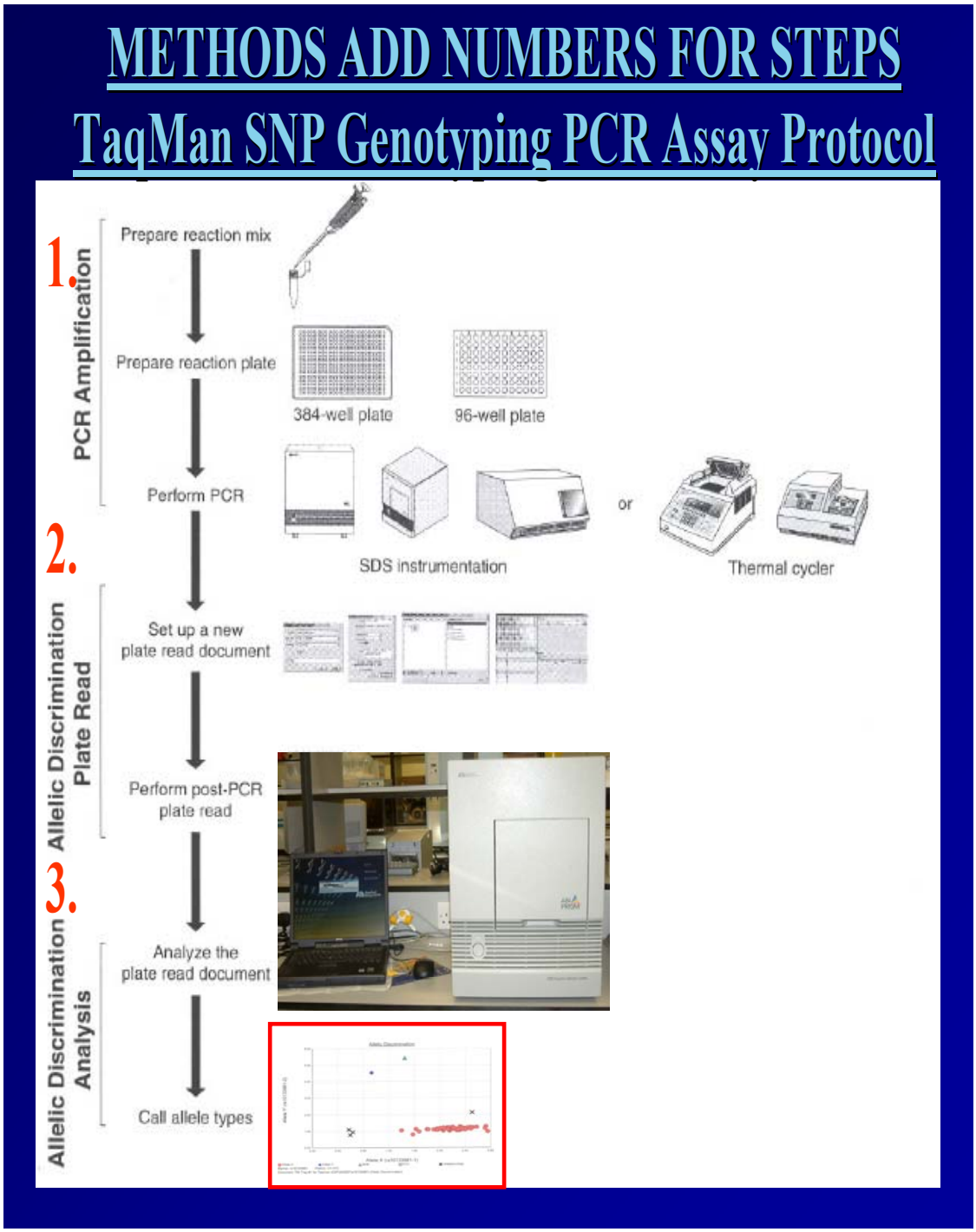


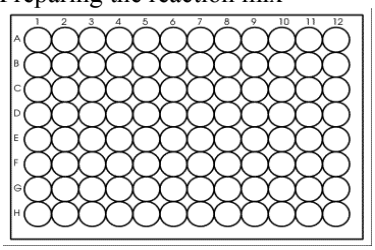
Figure 14. Methods Add Numbers for Steps; TaqMan SNP Genotyping PCR Assay Protocol

4.8.2.1 DNA extraction from blood and CSF is ready for TaqMan PCR amplication The purpose of the TaqMan PCR assay was to amplify specific target DNA sequences from the DNA

samples. The sequencing performance of the TaqMan PCR assay is of high quality with a failure rate of less than 1%, which equates to an incorrect assignment of less than one in every 2000 genotypes assayed (Ranade et al., 2001). The sequence detection system used for this procedure was an ABI Prism 7000. The TaqMan PCR assay included 1) DNA nucleotides, which served as the building blocks for the new DNA; 2) template DNA, which was the DNA sequence to be amplified; 3) primers, which were single-stranded DNA of about 20-50 nucleotides that were complimentary to a short region on either side of the template DNA; and 4) Taq polymerase, which is a heat stable enzyme that catalyzes new DNA synthesis (http://www.ncbi.nlm.nih.gov/). The steps of the TaqMan PCR process included the following: 1) elevate temperature to denature DNA strands (Table 9), 2) lower temperature to anneal primers (in molar excess compared to the concentration of DNA) (Table 10), 3) allow time for extension reactions to occur at optimum temperature (using Taq or other thermally-stable DNA polymerase) (Table 11), and 4) repeat the process 25-40 times in order to amplify more DNA for analysis (http://www.ncbi.nlm.nih.gov/).

An ideal primer has a stable 5' end and an unstable 3' end. The unstable 3' end limits bonding to false priming sites. The lower that this value is, the more likely the primer is to show secondary bands. The stable 5' end is called the GC clamp, which increases bonding to the target site. The lower that this value is, the more efficient the primer. The rating of a primer provides a quick way of measuring the predicted efficiency of a primer and of choosing between closely-matched primers. The higher the rating of a primer, the higher its amplification efficiency. The rating of a primer can be calculated as follows: Rating = 100 (DG (Dimer) * 1.8 + DG (Hairpin) * 1.4.

Table 9. TaqMan PCR Protocol Step I

<table>
<tr><th>Step</th><th colspan="2">Action</th></tr>
<tr><td>1</td><td>Preparing the reaction mix
  </td><td>a. Calculating the number of reactions to be performed for each assay.
b. Using the table below, calculate the volume of master mix components.
<table>
<tr><th>Reaction component</th><th>Volume/Well (5 ul volume reaction)</th></tr>
<tr><td>TaqMan universal PCR master mix No Amp Erase UNG(2X)</td><td>12.5/2</td></tr>
<tr><td>20 X Assays on demanal SNP genotyping Assay mix</td><td>1.25/2</td></tr>
<tr><td>Genomic DNA diluted in ddH2O</td><td>11.25/2</td></tr>
<tr><td>Total</td><td>25</td></tr>
</table>
<u>PCR mix</u>: <u>6.25 ul</u> * 96 (wells) = 600ul
<u>Assay</u>: <u>0.625 ul</u> * 96 (wells) = 60 ul
<u>Sterile water</u>: <u>5.5ul</u> * 96 (wells) = 528 ul
(sterile→ PCR→ Assay)
(600 ul + 60 ul + 528 ul) / 12 = 99 ul (for 99 ul / 8) = 12.5ul (for each plate)
c. Pipette the reagents into a sterile tube.</td></tr>
<tr><td>2</td><td>Preparing the reaction plate</td><td>a. Pipette 25 ul of reaction mix into each well for a 96-well plate.
b. Seal the plate.</td></tr>
<tr><td>3</td><td>Perform PCR</td><td>a. Program the thermal cycler with the PCR conditions.
b. Set the reaction volume to 25 ul for the 96-well plate.
c. Load the reaction plate into the thermal cycler.
d. Begin thermal cycling (40 times) then 10º C to cool down at the end.
e. for blood DNA extraction:
50º C (2’)->95º C (10’)->[95º C(15”)->60º C(1’)]40 cycle->10º C
for CSF DNA extraction
50º C (2’)->95º C (10’)->[95º C(15”)->58 ºC(1’30”)]40 cycle->10º C
<table>
<tr><td>Thermal Cycler</td><td colspan="4">Times and Temperatures</td></tr>
<tr><td rowspan="2">AB PRISM 7000 Sequence Detector</td><td>Stage 1</td><td>Stage 2</td><td colspan="2">Stage 3
Repeat 40 CYCLE</td></tr>
<tr><td>2 min
*50° C</td><td>10 min
*95° C</td><td>15 s
* 95° C</td><td>1 min
*60° C
1 min 30 s (58° C)</td></tr>
</table>
</td></tr>
</table>

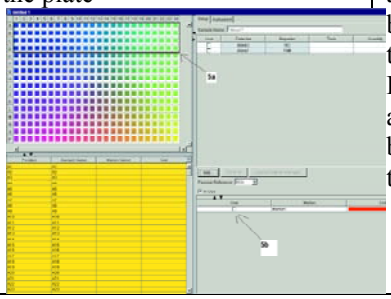
Moreover, the critical concerns about the outcome of the TaqMan assays are related to DNA quality, probe design, anticipated results, and the time and cost considerations (Hui, DelMonte, and Ranade, 2008). Because the SNPs are the most abundant and accessible class of polymorphisms present in the human genome, and because of the current popularity of using SNPs for genotyping, TaqMan has become the standard technique for DNA analysis.

4.8.2.2 Allelic discrimination plate read HapMap is the most popular human genome website that provides a haplotype map of the human genome and describes common patterns of human DNA sequence genetic variants from several populations: Yoruba in Ibadan, Nigerians, Japanese in Tokyo, Han Chinese in Beijing, China, and CEU Utah residents with ancestry from northern and western Europe (http://www.HapMap.org, 2007). Human DNA sequences have 99.9% similarity between two-unrelated people; the 0.1% genetic variants cause different responses to diseases or environmental risks. Each person has 24 pairs of chromosomes; the pair of alleles at a particular gene locus on these chromosome pairs called the genotype, may give rise to 10 million SNPs in a population. The set of alleles associated with SNPs in a particular region of a chromosome is called a haplotype. SNPs change the amino acid sequence within alleles, which changes the gene product and provides functional variations.

The SNPs for neuroglobin have been genotyped, which has revealed the cytogenetic map of chromosome 14, the two most useful SNPs tracks, allele positions, the frequency of each SNP characterized, and several reference sequence (rs) mRNA tracks (rs3783989, rs3783988, rs10133981, and rs7149300) that show the positions and structures of human protein-coding genes. In my 208 patients, there were 196 Caucasians (94.2%), 10 Blacks, and 2 American Indians or Alaskan Natives (1.0%). Therefore, rs3783988, which represented 57% A/A

nucleotide pairs, 41% A/G pairs, and 2% G/G pairs in the polymorphic site in the CEU, and rs10133981, which represented 90% G/G pairs, 10%G/T pairs, and 0% T/T pair were chosen to analyze genetic variants in the sample population.

Table 10. TaqMan PCR Protocol Step II

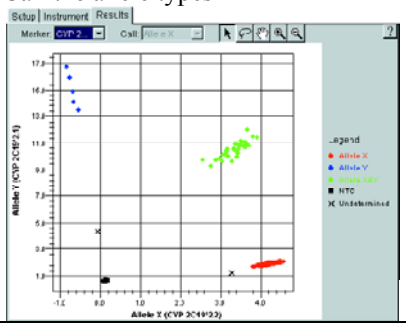
Step	Action	
1	Set up new plate read file	a. Launch the SDS software on the AB 7000 Prism computer. b. In the **New Document** window, create a new plate read file with the following attributes: - Under the **Assay** menu, select **Allelic Discrimination**. (rs3783988 & rs10133981) - Under the **Container** menu, select the **96-well plate** - Under the **Template** menu, select **Blank template**. c. Click **OK**. A new plate document opens with the appropriate attributes.
2	Create new markers	a. Open **Detector Manager** from the Tools menu. b. Click **File/New** to open the **Add Detector** dialog box and fill in the following information: - Type the name of allele 1, select **VIC** for reporter, and **Non-fluorescent** for quencher. - Type the name of allele 2, select **FAM** for reporter, and **Non-fluorescent** for quencher. c. Click **Done**. Then, the Add Detector dialog box closes. d. In the **Tools** menu, select Maker Manager and click Create Marker. The Add Marker dialog box opens. e. Click the **Enter name of new marker** to the field, type the new name, and click **OK**.
3	Apply detectors to the new marker	A marker must be configured with two detectors before it can be applied to a plate document. a. In the **Markers** text field, select the new marker. b. In the **Available Detectors** text field, click the detector (allele 1) marker c. Click **Add Detector**, repeat a through c for the second detector (allele 2) and click **Done**.
4	Select the sample wells and read the plate 	Select wells: a. Select the wells in the grid pane that contains the samples. b. Add the marker to those selected wells by checking the Use box adjacent to the desired marker in the **Set Up** window, and repeat for other wells. Read Plate: a. Save the document, and select the **Instrument** tab. b. Place the thermal cycled plate in the instrument, and select **Plate Read** on the instrument page. When the plate read has finished, save the file.

4.8.2.3 Allelic discrimination analysis The sequence detection systems from Applied Biosystems were used to measure the increase in the reporter fluorescence following PCR. Reporter signals were normalized to the emission of a passive reference (Applied Biosystems TaqMan Allelic Discrimination Protocol, 2007):

$$\textbf{Rn (allele 1)} = \frac{\textbf{Emission Intensity of Allele 1 Reporter}}{\textbf{Emission Intensity of Passive Reference}}$$

$$\textbf{Rn (allele 2)} = \frac{\textbf{Emission Intensity of Allele 2 Reporter}}{\textbf{Emission Intensity of Passive Reference}}$$

Table 11. TaqMan PCR Protocol Step III

Step	Action	
1	Open the plate document	a. If the plate document file is not open, select **Open** in the **File** menu. b. In the **Look in** text field, navigate to and select the plate document file. c. Click **Open**. The software displays the plate document file. d. In the **Analysis** menu, select **Analyze**.
2	Call the allele types 	a. Click the **Results** tab. The software displays the allelic discrimination plot. b. Use the lasso tool to select one cluster of data points. c. In the **Call** drop-down list, select the appropriate call and repeat for remaining clusters. d. Select **File/Export** and export the results table. X - wild type, homogeneous. Normal SNPs variation. Y- mutated, homogeneous. Abnormal SNPs variation. XY - heterogeneous. Carriers.

4.8.3 Data analysis plan

All data are presented as means, medians, modes, minimum and maximum values, and the skewness, kurtosis, and standard deviations were reported for all variables as appropriate. We anticipated that patients with SNP1 and SNP2 genetic variants for Ngb would have different

GCS scores on hospital admission after severe TBI compared to patients without these variants (wildtype), and that patients with the SNP1 and SNP2 genetic variants for Ngb would have different functional outcomes at 3, 6, 12, and 24 months following injury. The following data analyses were completed for each specific aim of this study:

Specific Aim #1: Determine the frequency of Ngb variants in the DNA extracted from the CSF and blood of patients with severe TBI. The Ngb gene variants were described as follows: For SNP1 (rs3783988), the frequencies of the C/C, C/T, and T/T nucleotide pairs were determined, and then we collapsed the frequencies for C/C and C/T together to designate the "presence of variant" (Vpresent) group; T/T was designated as the “absence of variant” (Vabsent) group. For SNP2 (rs10133981), the frequencies of T/T, G/T, and G/G were determined, and then we collapsed T/T with G/T together to designate the Vpresent group; G/G was designated as the Vabsent group. The frequencies of the Ngb variants were determined by descriptive frequency statistics.

Specific Aim #2: Determine the demographic and clinical characteristics of the patients based on the presence or absence of Ngb variants in the TBI population. Demographic characteristics consisted of age, race, gender, and past medical history, and the clinical characteristics included the cause of the injury. Independent T-tests, Pearson’s Chi-square tests, Fisher’s Exact tests, and Exact tests were used to analyze the continuous and categorical variables in order to compare the demographic and clinical characteristics of patients in the TBI population with and without the Ngb variants.

Specific Aim #3: Determine the relationship between the Ngb variants (present or absent) and the severity of the TBI as measured by the GCS administered on admission. The relationship between Ngb variants and GCS score were analyzed by Chi-square tests. ANOVAs were used to further evaluate differences in the GCS scores between different groups of Ngb variants. We also dichotomized the GCS into two subgroups (GCS 3-5, GCS 6-8) and analyzed the data for between-group differences in GCS score for the patients with and without Ngb variants using Pearson's Chi-Square test and Fisher's Exact test.

Specific Aim #4: Determine potential differences in the functional outcomes (GOS scores) at 3, 6, 12, and 24 months post injury for patients with and without Ngb variants (good outcome = GOS 4-5; poor outcome = GOS 1-3). We performed Chi-square analyses to determine if patients with or without the Ngb variants had differences in outcome (good or poor) at the stated time points. We also examined the outcomes assessed with the DRS and NRS when available. Lastly, we performed logistic and linear regression analyses in order to test the relationships between the SNP1 and SNP2 variants for Ngb and the three outcome measures.

4.9 LIMINATIONS TO THE RESEARCH PLAN

There is currently no research showing the existence of SNP sequence variants for Ngb in humans, making it difficult to interpret the results of the present study in terms of the findings of others. Additionally, the study population was limited to Caucasians and African Americans. HapMap studied normal individuals comprised of four racial groups, so comparisons of the HapMap data with the study population of brain-injured patients comprised of only 2 races is somewhat inexact. A sample that includes patients of other races would have enhanced

generalizability of the findings. Additionally, Xenon CT is a unique technique for measuring CBF, but this method was used for patients with TBI of the parent studies only during the years 2002-2005. Thus, only 21 patients had Xenon CT results available. The availability of more patients with Xenon CT results and, therefore, more CBF data would have provided more detailed information on the severity of hypoxia or ischemia in the samples.

5.0 RESULTS

This chapter summarizes the results of the analyses. First, the analyses of the total sample population are described; follow by the analyses for each of the specific aims. A total of 216 patients were available within the genetics study database (parent study), as described previously. Three of these patients were found to have a GCS > 8, meaning that they were enrolled in the study, but after enrollment they were found to not fulfill study entry criteria and were eliminated from the analyses. Also, 5 patients who were enrolled in the parent study did not have any demographic or TBI data available and were consequently eliminated from the analyses. Therefore, 208 patients from the parent study were found to be suitable for inclusion in the study.

5.1 TOTAL SAMPLE DESCRIPTION

5.1.1 Genotype variants in the sample of 208 patients

As previously described, the SNP1 (rs3783988) and SNP2 (rs10139881) variants of Ngb were selected for testing, and the frequencies for the genotype and genotype groupings in the sample of 208 patients is listed in Table 12. The genotyped frequencies for SNP1 included homozygous C/C (N = 9, 4.3%) and T/T (N = 128; 61.5%) nucleotide pairs, and heterozygous C/T (N = 68,

32.7%) pairs, with 3 patients (1.4%) for which the genotype was undetermined. The genotype frequencies for SNP2 include homozygous G/G (N = 187, 89.9%) and T/T (N = 2, 1.0%) nucleotide pairs and heterozygous G/T (N = 14, 6.7%) pairs, with 5 patients (2.4%) for which genotype was undetermined. SNP1 and SNP2 were further dichotomized into "Variant present" (Vpresent) and "Variant absent" (Vabsent) based upon the homozygote, heterozygote, or other-variant homozygote designations from the HapMap project (http://www.HapMap.org, 2008), which divides each genotype into wild typed or non-wild typed genetic variants.

Table 12. Total Sample Description (N = 208)

SNP1 (rs3783988)			SNP2 (rs10133981)		
	N	**Percent (%)**		**N**	**Percent (%)**
C/C	9	4.3%	**G/G**	187	89.9%
T/T	128	61.5%	**T/T**	2	1.0%
C/T	68	32.7%	**G/T**	14	6.7%
Present (C/C + C/T)	77	37%	**Present (T/T + G/T)**	16	7.7%
Absent (T/T)	128	61.5%	**Absent (G/G)**	187	89.9%
Missing	3	1.4%	**Missing**	5	2.4%

Key: SNP = single nucleotide polymorphism

Therefore, V present on SNP1 consisted of both C/C and C/T as non-wild typed variants, of which there were 77 patients (37%) with this designation. The SNP1 V absent group consisted of the T/T wild typed variant, which represented 128 patients (61.5%). Therefore, under this dichotomization, about 1/3 of the sample was SNP1 Vpresent and about 2/3 was SNP1 Vabsent. For SNP2, Vpresent was designated as T/T and G/T, which characterized only 16 patients (7.6%), whereas Vabsent was designated as G/G and characterized 187 patients (89.9%). Thus, the majority of the sample was SNP2 Vabsent. The genotype frequencies of 8 individuals could not be determined with the TaqMan PCR assay. Therefore, 3 patients had SNP1 data missing but

SNP2 data present, and 5 patients had SNP2 data missing but SNP2 data present. In summary, in this sample of 208 patients, more patients were likely to be Vabsent for both SNP1 and SNP2, although this finding was more pronounced for SNP2.

5.1.2 Demographic and clinical characteristics of the total sample of 208 patients

The demographic and clinical characteristics of the sample are indicated in Table 13. Overall, the patients in the sample were young, with a mean age of only 34.03 $\pm$ 14.45 years (mean $\pm$ SD; range 16–73 years). The sample curve describing age was normal in terms of its spread, with a skewness $\pm$standard error of skewness equal to 0.828 $\pm$ 0.168, which is within normal limits. The sample curve peak was also normal, with kurtosis $\pm$ standard error of kurtosis equal to -0.092 $\pm$ 0.334. For gender, slightly over three-fourths of the sample was male (N = 162; 77.9%) whereas a little less than a quarter (N = 44, 21.2%) was female. For race, the sample consisted primarily of Caucasians (N = 196, 94.7%; 154 males and 40 females), with a much smaller representation of African Americans (N = 10, 4.8%; 6 males and 4 females), American Indians, or Alaskan Natives (N = 2, 1.0%; 2 males).

The frequency analysis for past medical history was subdivided into seven major categories: no medical problems (N = 110, 52.9%), drug abuse (N = 6, 2.9%), hypertension (N = 4, 1.9%), pulmonary disease (N = 3, 1.4%), cardiac disease (N = 3, 1.4%), neurologic system disease (N = 1, 0.5%), and other medical problems (N = 32, 15.4%) such as diabetes mellitus. Therefore, the prevalence of past medical problems was very small in each category. This finding is consistent with the young age of the sample population.

The most common causes of injury in this population included: 1) motor vehicle accident (MVA) (N = 94, 45.2%), motorcycle accident (N = 35, 16.8%), fall (N = 33, 15.9%), all terrain

vehicle (ATV) crash (N = 11, 5.3%), pedestrian (N = 10, 4.8%), assault (N = 6, 2.9%), and other (N = 17, 8.2%). This information was missing for only 1 patient (0.5%). Therefore, the most prevalent cause of TBI in this population was MVA, followed by motorcycle accidents, and ATV crashes, with few pedestrian accidents or assaults. Most of the injuries in this sample were due to crashes of transport vehicles.

The distribution of the scores on the GCS on admission for the sample population was as follows: 11.1% GCS 3 (N = 23), 17.3% GCS 4 (N = 36), 13.9% GCS 5 (N = 29), 19.2% GCS 6 (N = 40), 31.7% GCS 7 (N = 66), and 6.7% GCS 8 (N = 14), with no missing data. Therefore, 88 patients (42.4%) had GCS scores ranging from 3-5, whereas 120 patients (56.6%) had GCS scores of 6-8. Therefore, this sample was fairly equally distributed between the two subgroups of GCS scores: poorer (scores 3-5) versus better (scores 6-8). The GCS distribution was found to be normal. The mode of GCS scores of the 208 patients with TBI was a score of 7. In summary, the sample was comprised of mostly young males, with few past medical problems, that were injured while riding in transportation vehicles.

Table 13. Total Sample Description-Demographic and Clinical Characteristics (N = 208)

Variable	Value
Age (Mean years ± SD)	34.03 ± 14.45
Gender (N = 208; 100%)	
Male	162 (77.9%)
Female	44 (21.2%)
Missing	2 (1.0%)
Race (N = 208; 100%)	
American Indian/Alaskan Native	2 (1.0%)
African American	10 (4.8 %)
Caucasian	196 (94.2%)
Medical History (N = 208; 100%)	
None	110 (52.9%)
Drug Abuse	6 (2.9%)
Hypertension	4 (1.9%)
Pulmonary Disease	3 (1.4%)
Cardiac Disease	3 (1.4%)
Neuro. Disease	1 (0.5%)
Other	32 (15.4%)
Missing	49 (23.6%)
Mechanism of Injury (N = 208; 100%)	
Motor Vehicle Accident (MVA)	94 (45.2%)
Motorcycle	35 (16.8%)
Fall	33 (15.9%)
All Terrain Vehicle (ATV)	11 (5.3%)
Pedestrians	10 (4.8%)
Assault	6 (2.9%)
Other	17 (8.2%)
Missing	1 (0.5%)
Admission Glasgow Coma Scale (GCS) (N = 208; 100%)	
3	23 (11.1%)
4	36 (17.3%)
5	29 (13.9%)
6	40 (19.2%)
7	66 (31.7%)
8	14 (6.7%)

5.1.3 Outcomes for the total sample of 208 patients

The primary outcome of interest in this study was the results of the assessments with the GOS (Table 14). The data for outcomes assessed with the DRS and the NRS were also available, but for fewer patients.

The GOS 3-month scores were available for 167 patients (80.3%). The distribution of GOS scores for the patients was as follows: 22.1% GOS 1 (N = 46), 7.2% GOS 2 (N = 16), 31.7% GOS 3 (N = 66), 15.4% GOS 4 (N = 32), and 3.8% GOS 5 (N = 8). When the 3-month GOS scores were dichotomized into good (GOS 4-5) and poor (GOS 1-3), the majority of the patients scored poorly (N = 127, 61.1%), with a minority of the patients scoring good (N = 40, 19.2%). The data for 19.7% of the sample (N = 41) was missing.

The GOS 6-month scores were available for 161 patients (77.4%). The distribution of the GOS scores for the patients was as follows: 22.1% GOS 1 (N = 46), 5.3% GOS 2 (N = 11), 21.3% GOS 3 (N = 44), 17.3% GOS 4 (N = 36), and 11.5% GOS 5 (N = 24). When the 6-month GOS scores were dichotomized, 60 patients (28.8%) were rated as good, whereas 101 patients (48.6%) were rated as poor, with data missing data for 47 patients (22.6%). Therefore, although the majority of patients still rated as poor as at 6 months, the prevalence of poor functional status declined over time (the percentage of patients categorized as GOS poor was 61.1% at 3 months, but only 49% at 6 months). These data show that some patients had achieved good outcome between 3 and 6 months after injury.

Table 14. Total Sample Description-Glasgow Outcome Scale from 3 to 24 Months (N = 208)

GOS Month and score	Frequency (percent)	GOS Month and score	Frequency (percent)
GOS 3rd Month		**GOS 3rd Month**	
(Completed N = 167; 80.3%)		**Poor (1-3)**	127 (61.1%)
1	46 (22.1%)	**Good (4-5)**	40 (19.2%)
2	15 (7.2%)	**Missing**	41 (19.7%)
3	66 (31.7%)		
4	32 (15.4%)		
5	8 (3.8%)		
Missing	41 (19.7%)		
GOS 6th Month		**GOS 6th Month**	
(Completed N = 161; 77.4 %)		**Poor (1-3)**	101 (48.6%)
1	46 (22.1%)	**Good (4-5)**	60 (28.8%)
2	11 (5.3%)	**Missing**	47 (22.6%)
3	44 (21.2%)		
4	36 (17.3%)		
5	24 (11.5%)		
Missing	47 (22.6%)		
GOS 12th Month		**GOS 12th Month**	
(Completed N = 141; 67.8%)		**Poor (1-3)**	83 (39.9%)
1	40 (19.2%)	**Good (4-5)**	58 (27.9%)
2	9 (4.3%)	**Missing**	67 (32.2%)
3	34 (16.3%)		
4	30 (14.4%)		
5	28 (13.5%)		
Missing	58 (27.9%)		
GOS 24th Month		**GOS 24th Month**	
(Completed N = 104; 50.0 %)		**Poor (1-3)**	59 (28.4%)
1	37 (17.8%)	**Good (4-5)**	45 (21.6%)
2	4 (1.9%)	**Missing**	104 (50.0%)
3	18 (8.7%)		
4	16 (7.7%)		
5	29 (13.9%)		
Missing	104 (50%)		

The GOS 12-month scores were available for 141 patients (67.8%). The distribution of 12-month GOS scores for the patients was as follows: 19.2% GOS 1 (N = 40), 4.3% GOS 2(N = 9), 16.3% GOS 3(N = 34), 14.4% GOS 4 (N = 31), and 13.5% GOS 5 (N = 28), with 27.9% categorized as good (N = 58) and 29.9% categorized as poor (N = 83). At the 24-month GOS follow-up (total N = 104; 50.0%), the scores were distributed as follows: 17.8% GOS 1 (N = 37), 1.9% GOS 2 (N = 4), 8.7% GOS 3 (N = 18), 7.7% GOS 4 (N = 16), and 13.9% GOS 5 (N = 29),

with data missing for 104 patients (50.0%). When the scores were dichotomized, 83 patients (39.9%) were classified as poor by the 12-month GOS score, whereas 59 patients (28.4%) were classified as poor by the 24-month GOS score. The percentage of missing data was increased for the 24-month sampling time point. Only half of the patients were still active participants in the study by the time the 24-month GOS score was reported, which may suggest that patients with better recovery withdrew from the study. Nevertheless, based upon our data, functional status showed the biggest improvement between 3 and 6 months, with little additional improvement thereafter.

Next, we evaluated the DRS scores. We first examined the DRS distribution for normality using the Shapiro-Wilk test (a significant value indicated normality), which demonstrated normality at all time points ($p < 0.001$ was obtained at 3, 6, 12, and 24 months). The data for the between-group differences in outcome status on the DRS is listed in Table 15. Scores on the DRS could potentially range from 0-29 (score 30 = death). When the DRS data were analyzed as an 11-category variable, several of the categories had less than 5 patients and many had no patients. Therefore, these data were treated as a continuous variable, utilizing mean scores as the metric. Analysis of the skewness and kurtosis characteristics of the data revealed values acceptable for normality (see Table 15). The mean $\pm$ standard deviation for the DRS scores over time were as follows: 15.42 ± 11.61 at 3 months (N = 164, 78.85%), 13.72 ± 12.25 at 6 months (N = 160, 76.92%), 13.25 ± 12.61 at 12 months (N = 138, 66.35%), and 14.10 ± 13.40 at 24 months (N = 105, 50.48%). All DRS scores had standardized values less than 3 ($Z < 3$) at all time points, meaning that their distribution was normal. As with the GOS scoring, the number of patients with available DRS scores decreased over time. When the mean DRS scores for available patients were closely examined, outcome status did not appear to change very much

over time. The reasons for missing data were that patients were cognitively or physically unable to respond, patients or their families refused to respond, patients gave less than their best effort, patients were in jail, patients resided too far away, or patients were lost to follow-up.

Table 15. Total Sample Description-Disability Rating Scale from 3 to 24 Months (N = 208)

DRS Month & Score	N	Range	Mean ± SD	Skewness/Kurtosis
DRS 3rd Month				
Valid	164 (78.85%)	0-30	15.42 ± 11.61	0.138/-1.685
Missing	44 (21.15%)			
DRS 6th Month				
Valid	160 (76.92%)	0-30	13.72 ± 12.25	0.319/-1.649
Missing	48 (23.08%)			
DRS 12th Month				
Valid	138 (66.35%)	0-30	13.25 ± 12.61	0.364/-1.673
Missing	70 (33.65%)			
DRS 24th Month				
Valid	105(50.48%)	0-30	14.10 ± 13.40	0.215/-1.853
Missing	103 (49.52%)			

The functional outcome measurement used was the NRS (Table 16). The histogram and QQ plot of the NRS scores at months 12 and 24 are represented by long tails on the higher scores, which indicates that few patients had worse recovery in terms of their functional outcome (the higher score the on the NRS, the worse the outcome status). The patients that worsened over time (12-month NRS: N = 6, score >70; 24-month NRS: N = 3, score > 50) were not excluded from the study because these outcomes had potential implications for interpretation and evaluation.

Additionally, each NRS score at months 3-24 had one patient with a standardized value above 3 ($Z > 3$): $Z = 3.48294$ for month 3, $Z = 3.62166$ for month 6, $Z = 3.4026$ for month 12, and $Z = 3.054$ for month 24); however, the patient at each time point was not excluded from the study because the Z value was not far beyond 3 standard deviations. This decision was justified

when the Shapiro-Wilk tests revealed that the NRS scores were normality distributed (a significant p value indicates normality), $p = 0.001$ at 3 months, $p = 0.002$ at 6 months, $p < 0.001$ at 12 months, and $p = 0.002$ at 24 months. We next evaluated the NRS scores over time and found that the means $\pm$ standard deviations over time were as follows: 40.63 ± 9.87 at 3 months (N = 62, 29.81%, range = 29-75), 41.13 ± 9.13 at 6 months (N = 76, 36.54%, range = 29-74), 41.65 ± 12.84 at 12 months (N = 71, 34.13%, range = 29-85), and 41.24 ± 9.23 at 24 months (N = 38, 18.27%, range = 31-69). Therefore, similar to findings with the DRS, mean NRS scores did not change much over time.

Table 16. Total Sample Description-Neurobehavioral Rating Scale from 3 to 24 Months (N = 208)

NRS Month & Score	N	Range	Mean $\pm$ SD	Skewness/Kurtosis
NRS 3rd Month				
Valid	62 (29.81%)	29-75	40.64 ± 9.87	1.488/2.053
Missing	146 (70.19%)			
NRS 6th Month				
Valid	76 (36.54%)	29-74	41.13 ± 9.13	1.151/1.186
Missing	132 (63.46%)			
NRS 12th Month				
Valid	71 (34.13%)	29-85	41.65 ± 12.84	1.590/2.100
Missing	137 (65.87%)			
NRS 24th Month				
Valid	38 (18.27 %)	31-69	41.24 ± 9.23	1.262/1.366
Missing	170 (81.73%)			

Next, we reviewed the total sample to determine if a correlation existed between the GCS scores and the outcome measures (GOS, DRS, and NRS) in order to examine the connection between TBI severity and outcomes which might also impact the results (Table 17). We first attempted to use the Exact test to evaluate the correlation between the GCS scores 3-8 and the GOS scores 1-5. However, the amount of data was too large and exceeded both the time and memory available for SPSS to complete this run. However, we were able to run the Exact test

when GCS scores 3-8 were defined as a categorical variable and GOS scores were dichotomized as poorer and good. This analysis demonstrated a positive significant correlation on months 3 ($p = 0.004$), 6 ($p < 0.001$), 12 ($p < 0.001$), and 24 ($p = 0.004$, respectively).

Table 17. Examination of the Relationship between Initial Glasgow Coma Scale and Outcomes (GOS, DRS, NRS) for the Total Sample from 3 to 24 Months (N = 208)

	Statistical Analysis	**3rd Month**	**6th Month**	**12th Month**	**24th Month**
GCS (3-8) vs. GOS (1-5)	Exact test	†	†	†	†
GCS (3-8) vs. GOS (Di)	Exact test	0.004*	0.001**	0.001**	0.004*
GCS (Di) vs. GOS (Di)	Pearson's Chi-square test	0.001**	0.001**	0.001**	0.001**
GCS (3-8) vs. DRS & NRS	ANOVA	0.001** 0.691	0.001** 0.475	0.001** 0.422	0.001** 0.059
GCS (Di) vs. DRS & NRS	Independent t-test	0.001** 0.884	0.001** 0.569	0.001** 0.160	0.001** 0.149

Key: † = could not be computed because there was insufficient memory, ** = significant p value < 0.001, * = significant p value < 0.005, Di = dichotomous.

We next ran the Pearson's Chi-square test with the dichotomized GCS scores (poorer and better) and the dichotomized GOS scores (poorer and good) and also saw a strongly positive relationship between these two variables at months 3 ($p < 0.001$), 6 ($p < 0.001$), 12 ($p < 0.001$), and 24 ($p < 0.001$). We then also used one-factor ANOVAs to evaluate the relationship between the GCS (3-8) scores and the DRS and NRS scores. The findings revealed significant relationships between the GCS (3-8) and the DRS scores at every time point ($p < 0.001$), whereas there was no significant relationship between the GCS (3-8) scores and the NRS scores at 3 months ($p = 0.691$), 6 months ($p = 0.475$), 12 months ($p = 0.422$), and 24 months ($p = 0.059$). These relationships were maintained when the dichotomized GCS scores were compared with the DRS and NRS scores, respectively, for month 3 ($p < 0.001$ and $p = 0.884$), month 6 ($p <$

0.001 and p = 0.569), month 12 (p < 0.001 and p = 0.160), and month 24 (p < 0.001, p = 0.149), using independent T-tests.

In summary, these findings indicate to us that there was an association between the severity of the TBI, as determined by the GCS scores on admission, and the functional outcomes assessed by the GOS at all time points. An association between TBI severity, as measured by the GCS and functional outcome was upheld for the DRS when this measure was examined as either a categorical or dichotomous variable, but there was no relationship between the GCS scores and the NRS-measured outcomes.

5.1.4 SNP variants and demographics in total sample of 208 patients

Using a total of 208 patients, we next examined whether or not differences existed in the patient demographics and clinical characteristics (Table 18) for patients in which the Ngb variants were present or absent.

For SNP1 and SNP2, there were no significant relationships between Vpresent/Vabsent and age (Independent t-test; SNP1 p = 0.517, SNP2 p = 0.482) or gender (Pearson's Chi-square for SNP1, p = 0.809; Fisher's Exact test for SNP2, p = 0.532). There was not a significant relationship for race (categorical variable; Exact test) for SNP1 (p = 0.767), but there was a significant relationship with race for SNP2 (categorical variable, p = 0.048). Because some experimental groups had less than 5 patients, we repeated the analysis using race as a dichotomized variable (non-Caucasian vs. Caucasian). Using Pearson's Chi-square for SNP1, the relationship was still not statistically significant (p = 0.359), whereas a significant relationship between SNP2 and race was maintained with the Fisher's Exact test (p = 0.045). Both medical history and cause of injury did not significantly differ for SNP1 (p = 0.133 and p = 0.655,

respectively) or SNP2 ($p = 0.822$ and $p = 0.992$, respectively), as assessed by the Exact tests. There was no significant relationship between the admission GCS score and the SNPs when GCS was utilized as a categorical variable with SNP1 ($p = 0.094$), a dichotomized variable with SNP1 ($p = 0.177$), a categorical variable with SNP2 ($p = 0.322$), or a dichotomized variable with SNP2 ($p = 0.543$).

Table 18. Total Sample Between-Group Difference of Demographics and Patient Characteristics for the Presence and Absence of Variants on SNP1 and SNP2 (N = 208)

	SNP1 (rs3783988)			SNP2 (rs10133981)		
	Present	**Absent**	**p**	**Present**	**Absent**	**p**
Age (mean ± SD)[T]	34.84 ± 15.35	33.49 ± 13.89	0.517[T]	31.31 ± 12.71	33.94 ±14.43	0.482[T]
Gender[C]			(N = 203)	14 (87.5%)		(N = 201)
Male	61 (79.2%)	98 (77.8%)	0.809[C]	2 (12.5%)	145 (78.4%)	0.532[F]
Female	16 (20.8%)	28 (22.2%)			40 (21.6%)	
Race[E]			(N = 205)			(N = 203)
American Indian Alaskan Native	1 (1.3%)	1 (0.8%)	0.767[E]	1 (6.3%)	1 (0.5%)	0.048[E]
African American	5 (6.5%)	5 (3.9%)		2 (12.5%)	7 (3.7%)	
Caucasian	71 (92.2%)	122 (95.3%)		13 (81.3%)	179 (95.7%)	
Race[C]			(N = 205)			(N = 203)
Non-Caucasian	6 (7.8%)	6 (4.7%)	0.359[C]	3 (18.8%)	8 (4.3%)	0.045[F]
Caucasian	71 (92.2%)	122 (95.3%)		13 (81.3%)	179 (95.7%)	
Medical History[E]		73 (74.5%)	(N = 157)			N = 157)
None	37 (62.7%)	3 (3.1%)	0.133[E]	9 (69.2%)	100 (69.4%)	0.822[E]
Drug Abuse	3 (5.1%)	1 (1.0%)		0 (0.0%	6 (4.2%)	
Hypertension	3 (5.1%)	0 (0.0%)		0 (0.0%)	4 (2.8%)	
Pulmonary Disease	3 (5.1%)	1 (1.0%)		0 (0.0%)	3 (2.1%)	
Cardiac Disease	1 (1.7%)	1 (1.0%)		0 (0.0%)	3 (2.1%)	
Neuro. Disease	0 (0.0%)	19 19.4%)		0 (0.0%)	1 (0.7%)	
Other	12 (20.3%)			4 (30.8%)	27 (18.8%)	
Cause of Injury[E]			(N = 204)			(N = 204)
Motor Vehicle Accident (MVA)	33 (43.4%)	60 (46.9%)	0.655[E]	8 (50.0%)	85 (45.7%)	0.992[E]
Motorcycle	14 (18.4%)	20 (15.6%)		2 (12.5%)	32 (17.2%)	
Fall	13 (17.1%)	20 (15.6%)		2 (12.5%)	28 (15.1%)	
All Terrain Vehicle (ATV)	3 (3.9%)	8 (6.3%)		1 (6.3%)	10 (5.4%)	
Pedestrians	2 (2.6%)	8 (6.3%)		1 (6.3%)	9 (4.8%)	
Assault	2 (2.6%)	4 (3.1%)		1 (6.3%)	5 (2.7%)	
Other	9 (11.8%)	7 (5.5%)		1 (6.3%)	16 (8.6%)	
Admission Glasgow Coma Scale (GCS)[E]			(N = 205) 0.094[E]			(N = 205) 0.322[E]
3	5 (6.5%)	18 (14.1%)		1 (6.3%)	22 (11.8%)	
4	20 (26.0%)	16 (12.5%)		5 (31.3%)	30 (16.0%)	
5	13 (16.9%)	16 (12.5%)		2 (12.5%)	26 (13.9%)	
6	12 (15.6%)	27 (21.1%)		1 (6.3%)	37 (19.8%)	
7	23 (29.9%)	41 (32.0%)		7 (43.8%)	58 (31.0%)	
8	4 (5.2%)	10 (7.8%)		0 (0.0%)	14 (7.5%)	
Admission Glasgow Coma Scale (GCS)[E]			0.177[C]			0.543[C]
Poor (3-5)[C]	38 (49.4%)	50 (39.7%)		8 (50.0%)	78 (42.2%)	
Better (6-8)	39 (50.6%)	76 (60.3%)		8 (50.0%)	107 (57.8%)	

Key: C = Pearson's Chi-square test; E = Exact test; F = Fisher's Exact test, T = Independent t-test.

Because the Pearson Chi-square test that was used to examine the relationship between the dichotomized race variable and the SNP allele frequencies was significant, we decided to exclude non-Caucasian patients (N = 12) from the subsequent analyses to eliminate the potential confound by race on SNP allele frequencies. Furthermore, the seven categorical domains of the medical history decreased the sensitivity of the Pearson Chi-square tests for both SNP1 and SNP2 due to very low numbers of patients in some of the experimental groups. Therefore, medical history was dichotomized into two categorical domains –significant medical history or non-significant medical history.

Thus, we limited the final sample to 196 Caucasian patients. Based on the elimination of non-Caucasians from further analyses and collapse of the variable categories as indicated, Table 19 now shows a condensed version of the medical history and cause of injury for the Caucasian patients. Specifically, the average age was 34.11 $\pm$ 14.57 years (mean $\pm$ SD), there were 154 males (78.6%) and 40 females (20.4%), 106 patients had no history of health problems, and 45 patients (23.0%) had a previous medical history. The majority of those patients experienced injury from a motor vehicle accident (N = 89, 45.4%), whereas the minority were injured from an assault (N = 4, 2.0%). Upon admission, 83 patients (42.3%) had a GCS score of 3-5 (poorer), whereas 112 patients (57.1%) had a GCS score of 6-8 (better).

Table 19. Total Sample Description-Demographic and Clinical Characteristics (N = 196)

Variable	Value
Age (Mean years ± SD)	34.11 ± 14.57
Gender (N = 196; 100%)	
Male	154 (78.6%)
Female	40 (20.4%)
Missing	2 (1.0%)
Medical History (N = 196; 100%)	
None	106 (54.1%)
Drug Abuse	6 (3.1%)
Hypertension	4 (2.0%)
Pulmonary Disease	2 (1.0%)
Cardiac Disease	3 (1.5%)
Neuro. Disease	1 (0.5%)
Other	29 (14.8%)
Missing	45 (23.0%)
Mechanism of Injury (N = 196; 100%)	
Motor Vehicle Accident (MVA)	89 (45.4%)
Motorcycle	34 (17.3%)
Fall	31 (15.8%)
All Terrain Vehicle (ATV)	11 (5.6%)
Pedestrians	10 (5.1%)
Assault	4 (2.0%)
Other	15 (7.7%)
Missing	1 (0.5%)
Admission Glasgow Coma Scale (GCS) (N = 196; 100%)	
3	23 (11.7%)
4	33 (16.8%)
5	27 (13.8%)
6	36 (18.4%)
7	64 (32.7%)
8	13 (6.6%)
Admission Glasgow Coma Scale (GCS) (N = 196; 100%)	
Poor (GCS 3-5)	83 (42.3%)
Better (GCS 6-8)	112 (57.1%)

5.2 SPECIFIC AIM 1: FREQUENCY OF NGB VARIANTS IN THE DNA EXTRACTED FROM THE CSF AND BLOOD OF PATIENTS WITH SEVERE TBI

Table 20 shows the data necessary to address Specific Aim #1 and indicates the frequency of the Ngb variants in this TBI sample population. The 196 Caucasians were characterized by the following nucleotide pairings: 4.1% C/C (N = 8), 62.2% T/T (N = 122), and 32.1% C/T (N = 63) for SNP1 (rs3783988), which was dichotomized to 36.2% SNP1 Vpresent (N = 71) and 62.2% SNP1 Vabsent (wild typed) (N = 122). For the SNP2 variant (rs10133981), the Caucasian patients were characterized as follows: 91.3 % G/G (N = 179), 0.5% T/T (N = 1), and 6.1% G/T (N = 12), which dichotomized to 6.6% SNP2 Vpresent (N = 13) and 91.3% SNP2 Vabsent (N = 179). Thus, the sample was predominantly absent for both variants of SNPs, but the effect was more pronounced for SNP2.

Table 20. Frequency and Percentage of Variants of SNP1 and SNP2 in 196 Caucasians

SNP1 (rs3783988)			SNP2 (rs10133981)		
	N	**Percent (%)**		**N**	**Percent (%)**
C/C	8	4.1%	**G/G**	179	91.3%
T/T	122	62.2%	**T/T**	1	0.5%
C/T	63	32.1%	**G/T**	12	6.1%
Present (C/C + C/T)	71	36.2%	**Present (T/T + G/T)**	13	6.6%
Absent (T/T)	122	62.2%	**Absent (G/G)**	179	91.3%
Missing	3	1.5%	**Missing**	4	2.0%

5.3 FIRST SECTION SPECIFIC AIM 2: RELATIONSHIP BETWEEN DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF PATIENTS BASED ON THE PRESENCE/ABSENCE OF NGB VARIANTS IN THE TBI POPULATION

Table 21 shows the results of our analyses examining the relationship between the demographic and clinical characteristics of the patients with TBI in the sample and the presence or absence of the variants of the SNPs. The data demonstrate that there was no significant relationship between the demographic and clinical characteristics of age, gender, medical history, or cause of injury and the presence or absence of variants on either SNP for the 196 Caucasian patients. We did not observe any trends in these data that would be of clinical interest.

Table 21. Demographics and Clinical Characteristics of 196 Caucasian Patients Based on the Presence or Absence of Ngb Variants on SNP1 and SNP2

	SNP1 (rs3783988)			SNP2 (rs10133981)		
	Present	**Absent**	**p**	**Present**	**Absent**	**p**
Age (mean ± SD)[T]	34.35 ± 15.49	33.34 ± 14.01	0.355[T]	32.23 ± 13.42	33.83 ±14.50	0.700[T]
Gender[C]			(N = 191)			(N = 190)
Male	57 (80.3%)	94 (78.3%)	0.749[C]	11 (84.6%)	140 (79.1%)	1.000[F]
Female	14 (19.7%)	26 (21.7%)		2 (15.4%)	37 (19.5%)	
Medical History[E]			(N = 149)			(N = 149)
None	34 (61.8%)	72 (76.6%)	0.173[E]	7 (70.0%)	98 (70.5%)	0.856[E]
Drug Abuse	3 (5.5%)	3 (3.2%)		0 (0.0%)	6 (4.3%)	
Hypertension	3 (5.5%)	1 (1.1%)		0 (0.0%)	4 (2.9%)	
Pulmonary Disease	2 (3.6%)	0 (0.0%)		0 (0.0%)	2 (1.4%)	
Cardiac Disease	1 (1.8%)	1 (1.1%)		0 (0.0%)	3 (2.2%)	
Neuro. Disease	0 (0.0%)	1 (1.1%)		0 (0.0%)	1 (0.7%)	
Other	12 (21.8%)	16 (17.0%)		3 (30.0%)	25 (18.0%)	
Cause of Injury[E]			(N = 192)			(N = 192)
Motor Vehicle Accident(MVA)	31 (44.3%)	57 (46.7%)	0.813[E]	7 (53.8%)	82 (46.1%)	0.958[E]
Motorcycle	13 (18.6%)	20 (16.4%)		2 (15.4%)	31 (17.4%)	
Fall	13 (18.6%)	18 (14.8%)		2 (15.4%)	26 (14.6%)	
All Terrain Vehicle (ATV)	3 (4.3%)	8 (6.6%)		1 (7.7%)	10 (5.2%)	
Pedestrians	2 (2.9%)	8 (6.6%)		1 (7.7%)	9 (5.1%)	
Assault	1 (1.4%)	3 (2.5%)		0 (0.0%)	4 (2.2%)	
Other	7 (10.0%)	7 (5.7%)		0 (0.0%)	15 (8.4%)	

Key: Ngb = neuroglobin, C = Chi-square test, E = Exact test, F = Fisher's Exact Test, T = Independent t-test.

5.4 SPECIFIC AIM 3: THE RELATIONSHI0P BETWEEN NGB VARAINTS (PRESENT/ABSENT) AND THE SEVERITY OF TBI AS MEASURED BY THE ADMISSION GCS

Table 22 shows that the data analyses for Specific Aim #3 in which the relationships between the presence or absence of the Ngb variants and the severity of TBI were examined, using the admission GCS as the metric. Our data indicate that the SNP1 variant was present in patients with GCS scores of 3 (N = 5, 7.0%), 4 (N = 19, 26.8%), 5 (N = 12, 16.9%), 6 (N = 10, 14.1%), 7 (N = 21, 29.6%), and 8 (N = 4, 5.6%).

Table 22. Glasgow Coma Scale Scores for 196 Caucasian Patients Based on the Presence or Absence of Neuroglobin Variants on SNP1 and SNP2

	SNP1 (rs3783988)			**SNP2 (rs10133981)**		
	Present	**Absent**	**p**	**Present**	**Absent**	**p**
Admission Glasgow Coma Scale (GCS)[E]			0.061[E]			0.497[E]
3	5 (7.0%)	18 (14.8%)		1 (7.7%)	22 (12.3%)	
4	19 (26.8%)	14 (11.5%)		3 (23.1%)	29 (16.2%)	
5	12 (16.9%)	15 (12.3%)		1 (7.7%)	25 (14.0%)	
6	10 (14.1%)	25 (20.5%)		1 (7.7%)	33 (18.4%)	
7	21 (29.6%)	41 (33.6%)		7 (53.8%)	57 (31.8%)	
8	4 (5.6%)	9 (7.4%)		0 (0.0%)	13 (7.3%)	
Poor (3-5)[C]	36 (50.7%)	47 (38.8%)	0.109[C]	5 (38.5%)	76 (42.7%)	0.765[C]
Better (6-8)	35 (49.3%)	74 (61.2%)		8 (61.5%)	102 (57.3%)	

Key: C = Chi-square test, E = Exact-test.

It appeared that more patients who were Vabsent for SNP1 had higher GCS scores than those who were Vpresent for SNP1. This finding approached but did not achieve statistical significance when GCS was examined as a categorical variable (p = 0.061). When we dichotomized the GCS scores into Poor or Better, there was a similar trend, but the result was not statistically significant (p = 0.109). The GSC scores of the SNP2 Vpresent patients were

distributed as follows: 7.7% GCS 3 (N = 1), 23.1% GCS 4 (N = 3), 7.7% GCS 5 (N = 1), 7.7% GCS 6 (N = 1), and 53.8% GCS 7 (N = 7). The GCS scores for the variants of SNP2 were not significantly different using the Exact test analysis (p = 0.497). The study further shows that the GCS 3-5 (poorer) condition in the SNP2 Vpresent variant was N = 5 (38.4%), and the GCS 6-8 (better) condition was N = 8 (61.5%), with no significant differences found for the SNP2 Vabsent group (p = 0.765). Briefly, only SNP1 variants showed an association with the severity of TBI on admission, with more patients with better GCS scores being SNP1 Vabsent.

5.5 SPECIFIC AIM 4: DIFFERENCES IN FUNCTIONAL OUTCOMES OF PATIENTS WITH AND WITHOUT NGB VARAINTS (PRESENT/ABSENT) OVER TIME

Specific Aim #4 was designed to determine if there were differences in the functional outcomes of patients, based on the presence or absence of the Ngb variants.

The results of the analyses of the GOS scores are listed in Table 23. The Pearson Chi-square test was used to assess differences in the GOS scores based on the presence or absence of Ngb variants in SNP1 and SNP2. For SNP1, when the GOS was maintained as a categorical variable, we found statistically significant differences for the 3-month GOS (N = 156, p = 0.015), the 6-month GOS (N = 151, p = 0.109), and the 12-month GOS (N = 133, p = 0.019) scores, but not for the24-month GOS score (N = 100, p = 0.242). However, when the GOS categories were dichotomized (good vs. poor), statistical significance was achieved at every time point. Although the findings were significant for both sets of variants, more patients characterized as SNP1 Vabsent were GOS good at 3 months (N = 30, 30.3%), 6 months (N = 44, 45.4%), 12 months (N

= 42, 51.9%), and 24 months (N = 34, 51.5%) post TBI than for the patients classified as SNP1 Vpresent (N = 8, 14.0%, p = 0.023; N = 13, 24.1%, p = 0.010; N = 13, 25.0%, p = 0.002; N = 10, 29.4%, p = 0.035, respectively). This finding of patients with SNP1 Vabsent being more likely to have a GOS good outcome (dichotomized) than the SNP1 Vpresent patients also held true at all subsequent time measurement points as well. Thus, patients identified as SNP1 Vabsent appeared to have good outcomes over time. For SNP2, no statistically significant differences were achieved for any of the analyses, regardless of whether or not the GOS scores were categorized or dichotomized.

Table 23. Functional Outcome of Glasgow Outcome Scale from 3 to 24 Months for 196 Caucasian Patients Based on the Presence or Absence of Neuroglobin Variants on SNP1 and SNP2

	SNP1 (rs3783988)			SNP2 (rs10133981)		
	Present	**Absent**	**p**	**Present**	**Absent**	**p**
3rd Month			(N = 167;			(N = 154;
1	23 (40.4%)	19 (19.2%)	80.3%)	2 (20.0%)	38 (26.4%)	78.6%)
2	5 (8.8%)	9 (9.1%)	0.015[E]	3 (30.0%)	11 (7.6%)	0.159[E]
3	21 (36.8%)	41 (41.4%)		3 (30.0%)	60 (41.7%)	
4	8 (14.0%)	22 (22.2%)		1 (10.0%)	29 (20.1%)	
5	0 (0.0%)	8 (8.1%)		1 (10.0%)	6 (4.2%)	
Poor (GOS 1-3)	49 (86.0%)	69 (69.7%)	0.023[C]	8 (80.0%)	109 (75.7%)	1.000[F]
Good (GOS 4-5)	8 (14.0%)	30 (30.3%)		2 (20.0%)	35 (24.3%)	
6th Month			(N = 161;			(N = 148;
1	20 (37.0%)	21 (21.6%)	77.4%)	3 (25.0%)	36 (26.5%)	75.5%)
2	4 (7.4%)	6 (6.2%)	0.109[E]	2 (16.7%)	8 (5.9%)	0.421[E]
3	17 (31.5%)	26 (26.8%)		4 (33.3%)	39 (28.7%)	
4	8 (14.8%)	26 (26.8%)		3 (25.0%)	31 (22.8%)	
5	5 (9.3%)	18 (18.6%)		0 (0.0%)	22 (16.2%)	
Poor (GOS 1-3)	41 (75.9%)	53 (54.6%)	0.010[C]	9 (75.0%)	83 (61.0%)	0.536[F]
Good (GOS 4-5)	13 (24.1%)	44 (45.4%)		3 (25.0%)	53 (39.0%)	
12th Month			(N = 141;			(N = 131;
1	17 (32.7%)	19 (23.5%)	67.8%)	1 (10.0%)	34 (28.1%)	66.8%)
2	4 (7.7%)	5 (6.2%)	0.019[E]	1 (10.0%)	8 (6.6%)	0.330[E]
3	18 (34.6%)	15 (18.5%)		5 (50.0%)	28 (23.1%)	
4	9 (17.3%)	19 (23.5%)		2 (20.0%)	25 (20.7%)	
5	4 (7.7%)	23 (28.4%)		1 (10.0%)	26 (21.5%)	
Poor (GOS 1-3)	39 (75.0%)	39 (48.1%)	0.002[C]	7 (70.0%)	70 (57.9%)	0.523[F]
Good (GOS 4-5)	13 (25.0%)	42 (51.9%)		3 (30.0%)	51 (42.1%)	
24th Month			(N = 104;			(N = 98;
1	16 (47.1%)	18 (27.3%)	50.0%)	1(14.3%)	32 (35.2%)	50.0%)
2	1 (2.9%)	3 (4.5%)	0.242[E]	1 (14.3%)	3 (3.3%)	0.343[E]
3	7 (20.6%)	11 (16.7%)		2 (28.6%)	16 (17.6%)	
4	4 (11.8%)	12 (18.2%)		2 (28.6%)	14 (15.4%)	
5	6 (17.6%)	22 (33.3%)		1 (14.3%)	26 (28.6%)	
Poor (GOS 1-3)	24 (70.6%)	32 (48.5%)	0.035[C]	4 (57.1%)	51 (56.0%)	1.000[F]
Good (GOS 4-5)	10 (29.4%)	34 (51.5%)		3 (42.9%)	40 (44.0%)	

Key: F = Fisher's Exact test, C = Chi-square test, E = Exact test, * = statistically significant.

Next, we examined the relationship between the presence or absence of the Ngb variants of the SNPs with the outcome measures assessed with the DRS treated as a continuous variable (Table 24). When we utilized the Independent t-test for between-group differences for SNP1 (Vpresent or Vabsent), we found significant effects on DRS score at 3 months ($p < 0.001$), 6

months (p = 0.005), 12 months (p = 0.014), and 24 months (p = 0.017), but we did not find statistical significance for between-group differences when comparing the SNP2 variants and DRS scores at any time point (p = 0.310, p = 0.311, p = 0.809, and p = 0.975, respectively).

Table 24. Functional Outcome on the Disability Rating Scale from 3 to 24 Months for 196 Caucasian Patients Based on the Presence or Absence of Neuroglobin Variants on SNP1 and SNP2

	SNP1 (rs3783988)			SNP2 (rs10133981)		
	Present	**Absent**	**p**	**Present**	**Absent**	**p**
DRS 3rd Month			(N = 153)			(N = 151)
	19.70 ± 10.88	12.82 ± 11.30	0.001**[T]	18.80 ± 10.27	14.96 ± 11.61	0.310[T]
DRS 6th Month			(N = 150)			(N = 147)
	17.19 ± 11.87	11.44 ± 11.88	0.005*[T]	16.75 ± 11.52	13.04 ± 12.15	0.311[T]
DRS 12th Month			(N = 131)			(N = 129)
	16.31 ± 11.95	10.81 ± 12.48	0.014*[T]	12.00 ± 11.27	13.00 ± 12.61	0.809[T]
DRS 24thMonth			(N = 101)			(N = 99)
	18.06 ± 13.23	11.47 ± 12.89	0.017*[T]	13.57 ± 12.33	13.74 ± 13.41	0.975[T]

Key: T = Independent t-test, ** = significant p< 0.001, * = significant p < 0.05.

Next, we examined the relationship between the presence and absence of the Ngb variants of the SNPs for the outcome measures assessed with the NRS scores treated as a continuous variable, using the Independent t-test at months 3, 6, 12, and 24 (Table 25). Mean scores for the SNP1 Vpresent and the SNP1 Vabsent groups were nearly identical, with no statistically significant differences achieved at any time point. Similarly, the mean scores for the SNP2 Vpresent and the SNP2 Vabsent groups were not different from each other at any time point.

Table 25. Functional Outcome on the Neurobehavioral Rating Scale from 3 to 24 Months for 196 Caucasian Patients Based on the Presence or Absence of Neuroglobin Variants on SNP1 and SNP2

	SNP1 (rs3783988)			SNP2 (rs10133981)		
	Present	**Absent**	**p**	**Present**	**Absent**	**p**
NRS 3rd Month			(N = 58)			(N = 58)
	41.80 ± 11.12	39.58 ± 9.61	0.463[T]	31.50 ± 0.71	40.55 ± 10.01	0.210[T]
NRS 6th Month			(N = 74)			(N = 72)
	40.90 ± 8.58	41.00 ±9.42	0.968[T]	41.75 ± 13.35	41.03 ± 9.08	0.881[T]
NRS 12th Month			(N = 70)			(N = 69)
	45.33 ± 15.01	40.02 ±11.74	0.116[T]	35.50 ± 6.46	42.15 ± 13.19	0.323[T]
NRS 24thMonth			(N = 38)			(N = 37)
	40.36 ± 9.11	41.59 ± 9.42	0.715[T]	36.67 ± 3.215	41.94 ± 9.45	0.348[T]

Key: T = Independent t-test.

Finally, we utilized a logistic regression (Table 26) to measure the regressor's independent contribution to variations in the dependent variable (GOS), a dichotomous variable. Age (continuous), gender (dichotomous), GCS (dichotomous), and SNP were assigned as the co-variants. In order to standardize the analysis, males, poorer GCS, and the genotype Vpresent were designated as the standardized statements, which were then compared to females, better GCS, and the genotype Vabsent. The model Chi-square value showed a good fit for these data for the four co-variances, consistent with the Hosmer and Lemeshow test. Table 26 shows the constant coefficients, p values, odd ratios, and 95% confidence intervals. For the GOS scores at 3 months for SNP1, age showed a significant negative association with GOS, which meant that younger patients had better functional outcomes. For gender, there were no significant findings at 3 months post injury. With GCS as a dichotomous variable, the data showed that poorer GCS was positively associated with poorer GOS ($p = 0.028$). These same findings occurred for the 6-month, 12-month, and 24-month time points. SNP1 Vabsent was a significant predictor of functional outcome at the 12-month time point after controlling for age, gender, and GCS score.

Table 26. Summary Logistic Regression between Predictors (Age, Gender, GCS [Dichotomous], and SNP1) with GOS for Months 3 to 24

		Age	Gender	GCS (Di)	SNP1
GOS 3rd Month	**β**	-0.075	-0.151	1.009	0.695
	Significant	0.001*	0.767	0.028*	0.144
	Odds Ratio	0.928	0.859	2.743	2.005
GOS 6th Month	**β**	-0.081	-0.720	1.908	0.663
	Significant	0.001*	0.148	0.001*	0.133
	Odds Ratio	0.923	0.487	6.740	1.940
GOS 12th Month	**β**	-0.057	-0.271	1.957	0.973
	Significant	0.001*	0.573	0.001*	0.028*
	Odds Ratio	0.944	0.763	7.076	2.645
GOS 24th Month	**β**	-0.064	-0.812	1.770	0.765
	Significant	0.001*	0.141	0.001*	0.141
	Odds Ratio	0.938	0.444	5.872	2.148

Key: β = unstandardized coefficients, Di = dichotomous, * = significant $p < 0.001$, GOS = Glasgow Outcome Scale, GCS = Glasgow Coma Scale.

Next we repeated the regression analyses with SNP2 (Table 27) and similar results were found: younger age and high GCS remained significant predictors of good outcome, but there were no significant effects of SNP2 at any time point. The overall regression line was as follows: (Y) = β + β1 * (age) + β2 * (gender) + β3 * (GCS) + β4 * (SNP1 or SNP2). As a result, the best fit for this model was to include all variables. In summary, SNP2 was not a significant predictor for functional outcome as measured by the GOS after controlling for age, gender, and GCS score.

Table 27. Summary Logistic Regression between Predictors (Age, Gender, GCS [Dichotomous], and SNP2) for GOS for Months 3 to 24

		Age	Gender	GCS (Di)	SNP2
GOS 3rd Month	**β**	-0.80	-0.110	1.289	0.483
	Significant	0.001*	0.831	0.006	0.589
	Odds Ratio	0.923	0.896	3.629	1.621
GOS 6th Month	**β**	-0.083	-0.713	2.187	1.060
	Significant	0.001*	0.160	0.001*	0.189
	Odds Ratio	0.920	0.490	8.925	2.886
GOS 12th Month	**β**	-0.064	-0.151	2.277	1.061
	Significant	0.001*	0.756	0.001*	0.185
	Odds Ratio	0.938	0.859	9.747	2.890
GOS 24th Month	**β**	-0.068	-0.773	2.047	0.113
	Significant	0.001*	0.168	0.001*	0.898
	Odds Ratio	0.935	0.462	7.743	1.120

Key: β = unstandardized coefficients, Di = dichotomous,* = significant $p < 0.001$, GOS = Glasgow Outcome Scale, GCS = Glasgow Coma Scale.

Next, we applied the linear regression model to the DRS and NRS data for months 3, 6, 12, and 24, with age, gender, GCS (dichotomous), and SNP as co-variants. The standardized residual graphs of the histogram and normal P-P plot of the regression showed that the DRS and NRS scores were normally distributed. In Pearson's correlation matrix for all predictors, DRS scores at months 3, 6, 12, and 24 were 36%, 37%, 35%, and 36%, respectively. Table 28 shows the correlations between age, gender, GCS, and SNP1 for the DRS scores on months 3, 6, 12, and 24. For the age variable, older patients had higher DRS scores, which meant lower functional outcomes ($p < 0.001$) at every time point. Gender was a significant predictor of outcome at 3 and 6 months, but not at 12 or 24 months post injury. GCS score significantly predicted outcome in this model for months 12 and 24 (both $p < 0.001$). SNP1 was associated with outcome on the DRS for months 3 and 6 (both $p < 0.001$), but not for months 12 and 24. When beta (β) was negative, SNP1 Vpresent predicted a worse outcome on the DRS.

Table 28. Summary Linear Regression between Predictors (Age, Gender, GCS [Dichotomous], and SNP1) with DRS for Months 3 to 24

		Age	Gender	GCS (Di)	SNP1
DRS 3rd Month	**β**	0.283	1.803	-9.694	-4.464
	Significant	0.001*	0.001*	0.324	0.001*
	95% CI	0.181-0.384	-1.796-5.402	-12.807-(-6.581)	-7.630-(-1.298)
	R^2	0.365			
DRS 6th Month	**β**	0.305	1.954	-10.616	-3.071
	Significant	0.001*	0.001*	0.322	0.001*
	95% CI	0.198-0.413	-1.935-5.843	-13.963-(-7.268)	-6.467-0.324
	R^2	0.358			
DRS 12th Month	**β**	0.318	1.565	-10.610	-2.803
	Significant	0.001*	0.461	0.001*	0.141
	95% CI	0.197-0.440	-2.624-5.754	-14.336-(-6.883)	-6.543-0.938
	R^2	0.345			
DRS 24th Month	**β**	0.327	1.441	-11.095	-3.813
	Significant	0.001*	0.568	0.001*	0.106
	95% CI	0.187-0.467	-3.555-6.436	-15.616-(-6.573)	-8.451-0.824
	R^2	0.355			

Key: β = unstandardized coefficients, * = significant $p < 0.001$, R^2 = square of R, CI = confidence interval, DRS = Disability Rating Scale, GCS = Glasgow Coma Scale.

Similar to SNP1, the linear regression findings for SNP2 and DRS scores at months 3, 6, 12, and 24 showed that all Pearson's correlations were between 36%-38% (Table 29). Both age and GCS score were positively correlated with DRS at months 3 to 24 ($p < 0.001$), with younger patients and those patients with better GCS scores having better DRS scores in the model. Gender was not significantly correlated with DRS score. SNP2 was a significant predictor of DRS score at month 3 ($p = 0.037$) but not at the other time points. In conclusion, using the unstandardized coefficients of age, gender, GCS, and SNP2, the overall regression line was (Y) = β + β1 * (age) + β2 * (gender) + β3 * (GCS) + β4 * (SNP1or SNP2). As a result, the best fit for this model was to include all variables.

Table 29. Summary Linear Regression between Predictors (Age, Gender, GCS [Dichotomous], and SNP2) with GOS for Months 3 to 24

		Age	Gender	GCS (Di)	SNP2
DRS 3rd Month	**β**	0.292	1.920	-11.260	-6.529
	Significant	0.001*	0.302	0.001*	0.037*
	95% CI	0.189-0.394	-1.745-5.586	-14.383-(-8.137)	-12.647-(-0.412)
	R^2	0.355			
DRS 6th Month	**β**	0.309	1.929	-11.727	-5.291
	Significant	0.001*	0.334	0.001*	0.076
	95% CI	0.200-0.417	-2.004-5.862	-15.050-(-8.403)	-11.146-0.564
	R^2	0.357			
DRS 12th Month	**β**	0.339	1.086	-11.640	-1.831
	Significant	0.001*	0.621	0.001*	0.589
	95% CI	0.218-0.459	-3.135-5.307	-15.360-(-7.921)	-8.529-4.867
	R^2	0.347			
DRS 24th Month	**β**	0.346	1.056	-12.521	-1.662
	Significant	0.001*	0.680	0.001*	0.701
	95% CI	0.205-0.487	-4.018-6.130	-17.068-(-7.975)	-10.228-6.903
	R^2	0.354			

Key: β = unstandardized coefficients, * = significant $p < 0.001$, R^2 = square of R, CI = confidence interval, DRS = Disability Rating Scale, GCS = Glasgow Coma Scale.

Next, we utilized the predication models for the outcome measured by the NRS (Table 30). For SNP1, the Pearson's correlations were only 10%-22% for each of the time points examined. Age was the only variable that was a significant predictor of outcome on the NRS at 6 months ($p < 0.001$), 12 months ($p = 0.006$), and 24 months ($p = 0.035$). Because the NRS was completed for very few patients at months 3 to 24, the sample may have been too small to obtain robust findings for this variable. SNP1 had a negative beta (β), which indicated a trend towards better outcomes with SNP1 Vabsent, but this trend did not achieve statistical significance. The findings for SNP2 were similar to SNP1 in that age was the only variable that was a significant predictor of outcome. However, these findings are called into question because of the very small sample of patients with NRS data (Table 31).

Table 30. Summary Linear Regression between Predictors (Age, Gender, GCS [Dichotomous], and SNP1) with NRS for Months 3 to 24

		Age	Gender	GCS (Di)	SNP1
NRS 3rd Month	**β**	0.219	-1.716	0.142	-1.143
	Significant	0.064	0.612	0.964	0.711
	95% CI	-0.013-0.450	-8.460-5.027	-6.151-6.434	-7.301-5.015
	R^2	0.075			
NRS 6th Month	**β**	0.324	-4.621	0.397	0.456
	Significant	0.001*	0.065	0.869	0.837
	95% CI	0.158-0.489	-9.542-0.299	-4.397-45.190	-3.946-4.857
	R^2	0.203			
NRS 12th Month	**β**	0.372	-2.043	1.939	-3.736
	Significant	0.006*	0.564	0.589	0.259
	95% CI	0.112-0.631	-9.084-4.998	-5.200-9.078	-10.288-2.817
	R^2	0.161			
NRS 24th Month	**β**	0.252	-0.757	3.520	1.245
	Significant	0.035*	0.845	0.300	0.719
	95% CI	0.019-0.485	-8.578-7.063	-3.281-10.322	-5.724-8.214
	R^2	0.181			

Key: β = unstandardized coefficients, * =significant $p < 0.001$, R^2 = square of R, CI = confidence interval, NRS = Neurobehavioral Rating Scale, GCS = Glasgow Coma Scale.

Table 31. Summary Linear Regression between Predictors (Age, Gender, GCS [Dichotomous], and SNP2) with NRS for Months 3 to 24

		Age	Gender	GCS (Di)	SNP2
NRS 3rd Month	**β**	0.224	-2.063	0.033	7.439
	Significant	0.056	0.537	0.992	0.313
	95% CI	-0.006-0.454	-8.726-4.601	-6.426-6.491	-7.113-21.812
	R^2	0.097			
NRS 6th Month	**β**	0.353	-5.160	0.012	-.2.314
	Significant	0.001*	0.039*	0.996	0.593
	95% CI	0.185-0.522	-10.063-(-0.257)	-4.877-4.900	-10.918-6.290
	R^2	0.229			
NRS 12th Month	**β**	0.432	-2.279	0.105	-5.283
	Significant	0.001*	0.522	0.977	0.404
	95% CI	0.173-0.692	-9.342-4.784	-7.220-7.431	-7.282-17.847
	R^2	0.168			
NRS 24th Month	**β**	0.300	-1.807	1.533	5.088
	Significant	0.014*	0.608	0.660	0.334
	95% CI	0.066-0.534	-8.917-5.303	-5.490-8.557	-5.478-15.654
	R^2	0.227			

Key: β = unstandardized coefficients, * = significant $p < 0.001$, R^2 = square of R, CI = confidence interval.

Thus, the regression models demonstrated that when controlling for age, gender, and GCS, SNP1 Vabsent was not a good predictor of good functional outcome on the GOS except for month 12. However, SNP1 Vabsent was a predictor of better outcome on the DRS after controlling for the covariates at 3 and 6 months, but the effects were not statistically significant. The findings for SNP2 with this regression model were inconsistent, which is not surprising given that the number of patients with SNP2 Vabsent was very small (Table 32).

Finally, since the patient's medical history may be a factor in functional outcome following TBI; we developed the same series of models as described above but with medical history added as an additional controlling variable. However, medical history was not a significant predictor of outcome in any model or at any time point (data not shown).

6.0 DISCUSSION

6.1 SPECIFIC AIM 1: FREQUNCY OF NGB VARAINS IN THE DNA EXTRACTED FROM THE SCF AND BLOOD OF PATIENTS WITH SEVERE TBI

The only information regarding the frequency of Ngb variants was found from the HapMap database, which showed Ngb is located on chromosome 14. The number of locus link genes per 500-kb window across the chromosome is about 15, and the number of genotyped SNPs per 500-kb window across the chromosome is about 100. SNP1 (rs3783988) with the A/G nucleotides on the alleles has a negative strand relative to the human reference sequence (http://www.HapMap.org, 2008). For SNP1, the HapMap data are closest to the Ngb variants on chromosome 14, positions 76804333-76804333 of the cytogenetic chromosome bands in the HapMap gene bank. The HapMap description of the Caucasian population was similar to their Utah residents with ancestry from northern and western Europe (CEPH or CEU), which demonstrates genotype frequency of reference-homozygote (A/A [56.7%]), heterozygote (A/G [41.7%]), and other variant homozygote frequency (G/G [1.7%]) from the healthy population (Table 32).

In our study, the genotype frequencies of SNP1 were 4.0% C/C (as G/G; N = 8), 62.1% T/T (as A/A; N = 123), and 32.3% C/T (as A/G; N = 64). SNP1 Vpresent (C/C and C/T combined) can be classified as the non-wild typed variants. In contrast, SNP1 Vabsent (T/T) was

considered wild typed. Thus, when comparing our data to the HapMap data, HapMap for SNP1 in Caucasians was 43.3% SNP1Vpresent and 56.7% SNP1 Vabsent. This finding is comparable to our finding of 34.6% SNP1 Vpresent and 62.1% SNP1 Vabsent. Therefore, the HapMap genotyped frequencies for Caucasians are comparable to the sample frequencies of our Caucasian patients with a TBI, with the majority being SNP1 Vabsent (57% in HapMap vs. 63% in our sample) and the minority being SNP1 Vpresent (43% in HapMap vs. 37% in our sample). This information suggests that, firstly, our laboratory methods were probably accurate because we had similar findings for the primary variant. However, because HapMap is based on generally healthy individuals, our finding of a higher percentage of SNP1 Vabsent suggests that this variant may become more frequent under conditions of TBI. One possible explanation is that the SNP1 variant may code a functional region and also a linkage disequilibrium mark in SNP1.

Conversely, SNP2 is on chromosome 14 with positions 76805546-76805546 in the cytogenetic chromosome bands and has a positive strand relative to the human reference sequence. In the HapMap sample of Caucasians, the three genotyped frequencies were 90% G/G, 10% G/T, and 0.0% T/T, which is comparable to our TBI sample with 91.4% G/G (N = 181), 0.5% T/T (N = 1), and 6.1% G/T (N = 12). When dichotomized, SNP2 was Vabsent for 90% of Caucasians in HapMap and 93% in our sample; SNP2 was Vpresent for 10% of Caucasians in HapMap and 7% in our sample. This information suggests that our SNP analysis methods were accurate and thus comparable to the HapMap findings. The other important finding is that there were no meaningful differences between The HapMap frequency data and ours (only a 3% difference). This suggests that SNP2 may not vary in its frequency regardless of whether people are healthy or have a TBI. Another explanation could be that, since SNP2 is further from the Ngb reference region on the chromosome, SNP2 may be less likely to change under different health

conditions. Perhaps SNP2 is located in introns area, which cannot maintain any genotyped information or function.

We discovered a significant relationship between race and SNP2 when we included the 12 African American patients in our sample, which ultimately prompted our decision to exclude non-Caucasians from our analyses. When examining the HapMap data, the SNP1 prevalence for Yorubi Nigerians and Caucasians was more similar (57% Yorubi Nigerians SNP1 Vabsent vs. 62% Caucasians Vabsent) than the racial prevalence rates for SNP2 (30% Yorubi Nigerians SNP2 Vabsent vs. 93% Caucasian Vabsent). The differences in the prevalence distributions for SNP2 probably contributed to our significant findings for race and SNP2, which supported our decision to eliminate non-Caucasians from our outcome analysis because variance due to ancestry would have confounded our findings.

Table 32. Comparison of the Percentage of Variants on SNP1 and SNP2 in HapMap Data and Current Study Data

	SNP1 (rs3783988)			**SNP2 (rs10133981)**	
	HapMap Caucasian	**Current Study Sample Caucasians**		**HapMap Caucasian**	**Current Study Sample Caucasians**
Present (C/C + C/T)	43%	37%	**Present (T/T + G/T)**	0.1%	7%
Absent (T/T)	57%	63%	**Absent (G/G)**	90%	93%

6.2 SPECIFIC AIM 2: RELATIONSHIP BETWEEN DEMOGRAPHIC AND LICNIAL CHARACTERISITCS OF PATIENTS BASED ON THE PRESENCE/ ABSENCE OF NGB VARIANTS IN THE TBI POPULATION

We examined the relationship between age, gender, and medical history as our demographic characteristics (after eliminating race as described above) and cause of injury as our clinical characteristics for comparisons with the SNP1 and SNP2 variants. We found no significant relationships on the demographic variables and the SNP variants. SNP1 Vabsent patients were slightly younger (33 years) than the SNP1 Vpresent patients (35 years), but there were no between-group differences for the other demographic characteristics. Neither did we find any significant relationships between the demographics and SNP2. A possible explanation is that chromosome 14, where these SNPs are located, is not influenced by gender. Medical history also did not significantly vary with the SNP variants; however, the frequency of any significant past medical history was very low in this fairly young sample population. Analysis of the clinical characteristic of the mechanism of injury also did not reveal any significant relationship with SNP variants; however, because approximately 70% of the patients were injured by MVA, there may not be enough patients represented in the other injury groups to detect any significant effects of this variable. Also, the mechanism of injury and genotype may be unrelated.

6.3 SPECIFIC AIM 3: THE RELATIONSHIP BETWEEN NGB VARAINTS (PRESENT/ABSENT) AND THE SEVERITY OF TBI AS MEASURED BY THE ADMISSION GCS

GCS showed a strong association with SNP1, with GCS as a categorical variable (p = 0.061), and a non-significant trend when treated as a dichotomous variable (p = 0.109). Figure 15 shows that patients with SNP1 Vpresent were more likely to have an initial GCS score of 3-5 (poorer), whereas patients with SNP1 Vabsent were more likely to have an initial GCS score of 6-8 (better). However, the effect was different for SNP2 (Figure 16).

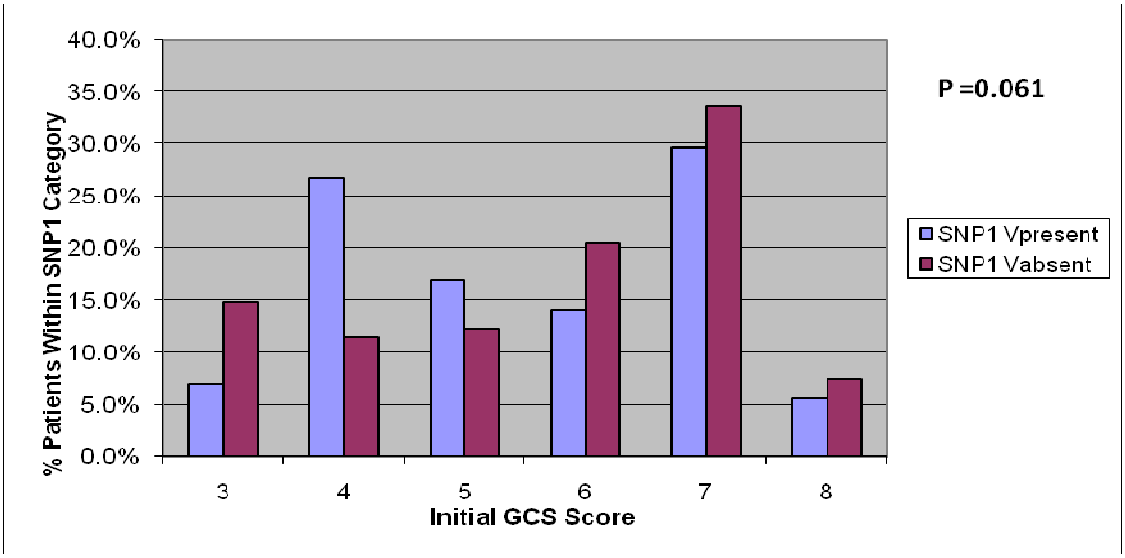


Figure 15. Relationship Between SNP1 Ngb Variants and Admission GCS Scores

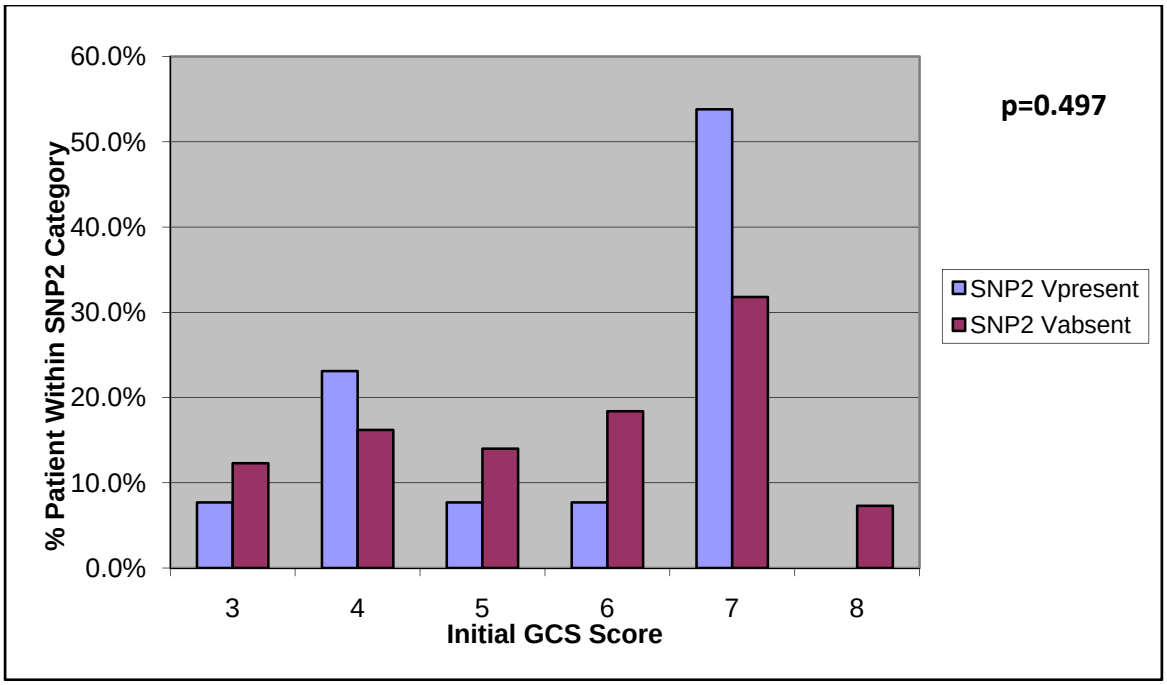


Figure 16. Relationship between SNP2 Ngb Variants and Admission GCS Scores

One possible explanation is that the characteristic of being SNP1 Vabsent somehow impacts the severity of the injury measured on admission (which can be hours after the insult). Possibly, having a genetic predisposition to SNP1 Vabsent may help the patient's brain to be better able to withstand a traumatic hypoxic or ischemic insult. The effect of the SNP1 Vabsent variant on Ngb (e.g., Ngb quantity, composition or behavior) in human patients with TBI is not yet known. The only literature available for comparison is a study in which Ngb was related to the severity of injury in an animal model of stroke.

Several studies with animal models of stroke have been conducted. According to Greenberg, Jin, and Khan (2008), Ngb is thought to have two possible modes of action in the cerebral hypoxia or ischemia animal model of stroke: 1) expression, or 2) protective adaptation during the acute period (Greenberg, Jin, and Khan, 2008). Greenberg's first study (2001) utilized 10 Sprague-Dawley male rats (290-320g; 5 experimental, 5 sham). He and his colleagues

developed an oligodeoxynucleotide (ODN), the design of which was based on the mouse Ngb sequence, and injected anti-ODN into the cerebral neurons of mice in order to overexpress Ngb 3 hours before a hypoxic state was induced. They also cloned mouse Ngb cDNA from hippocampal neurons of these same animals. Then, they created an experimental hypoxic model by subjecting the mice to MCAo for 90 min followed by reperfusion for 4-24 hr. Thus, focal ischemia was induced over an entire hemisphere. The animals were sacrificed and examined after 24 hr and assessed for both mRNA and the Ngb protein itself in the cytoplasm of neurons. Comparisons were made between the ischemic and non-ischemic hemispheres within each mouse using Western blots and immunocytochemistry. They found that Ngb mRNA was positively expressed after 24 hr in the brain tissue of animals that had been ODN-treated before the ischemic insult ($p < 0.02$) and that Ngb protein was increased in neurons of the cerebral cortex and hippocampus of the experimental animals, which makes sense because these areas are the most sensitive to ischemia. They also found that infarct size was decreased by 49-52% in the experimental animals.

In another study by this same group (Sun et al. 2003), Ngb expression was either reduced in Sprague-Dawley rats by administering Ngb anti-sense ODN or increased by intracerebral administration of an Ngb-expressing adeno-associated virus (AVV) vector. They then induced focal cerebral ischemia by MCAo for 90 min followed by reperfusion. The effects of the treatments were subsequently assessed by histological examination. They found that infarct size was increased by 56-60% in the group that received the antisense Ngb knockdown (the ODN group [$p < 0.02$]).

Similarly, and more recently, Wang et al. (2008) compared infarct volume for Ngb overexpressing transgenic mice (Ngb-Tg) to wildtype controls. They found that, compared with

the wild typed controls, the Ngb-Tg mice had significantly reduced brain infarction volumes at 24 hr after transient focal cerebral ischemia ($p < 0.05$), with the infarct reductions primarily occurring in the cortex. They found that the infarct volume was significantly decreased in the Ngb-Tg mice at 14 days post injury ($p < 0.05$).

In contrast, there have been some experiments which have not revealed such positive relationships between the degree of injury and Ngb levels. Hundahl et al. (2006) had somewhat different findings. In their experiment, spontaneously hypertensive male rats (SHRs) were randomly selected for 90 min of MCAo (N = 6) or sham surgery (N = 4; control) and euthanized after 24 hr. The animals were not pretreated in any way so as not to affect Ngb production. They examined Ngb mRNA in both groups of rats by Quality Reverse Transcription Polymerase Transcription and found significantly less Ngb mRNA in the ischemic hemispheres after 24 hr compared to controls ($p < 0.02$). They repeated the experiment using Wistar and Sprague-Dawley rats but failed to find Ngb expression. However, in two more animal groups which experienced the same procedures (MCAo [N = 7] or sham surgery [N = 6]) but with a survival period of 1 week, they discovered a negative correlation ($r = -0.85$) between infarct volume and Ngb expression, thus they found less Ngb in the ischemic hemispheres. The conclusion of their studies was that there was no difference in Ngb expression levels between ischemic and non-ischemic hemispheres. Shang et al. (2004) induced global ischemia in the gerbil forebrain, specifically in the cerebral cortex and hippocampus. The MCA was occluded for 90 min and CBF had decreased. The gerbils were then sacrified, and subsequent analyses revealed that Ngb protein and mRNA increased initially but then normalized within 10-20 min of the onset of reperfusion.

Schmidt, Haberkamp, Schmitz, Hankeln, and Burmester (2006) found that focal ischemia did not alter the expression of Ngb in spontaneously hypertensive rats. Another report by Shang et al. (2006) revealed that global ischemia did not alter the expression of Ngb in rat brains. Other studies (Fordel et al., 2004; Hundahl et al. 2005; Mammen et al., 2002) also showed that acute and chronic ischemia did not change the expression of Ngb mRNA or protein, but that Purkinje cells were the most sensitive to ischemia. No significant Ngb expression was found after chronic anoxia or hypoxia ($10\%O_2/90\%N_2$ for 2 weeks) (Brunori & Vallone, 2006).

In summary, our finding that a genetic variant of Ngb was related to better GCS scores seems to imply that individuals who may be capable of expressing Ngb of either increased quantity or better quality are better able to withstand the deleterious effects of a head injury. These findings are closest to the findings of Greenberg et al. who engineered animals to either increase or decrease the expression of Ngb. The lack of positive findings in the other studies where ischemia was induced irrespective of a predisposition to express Ngb is more difficult to explain. In these other studies, random animals were utilized that may or may not have had a genetic predisposition favoring their ability to increase Ngb production. Also, there were differences in the brain regions affected by the ischemic insult and the length of time the animals underwent ischemia. There may have been differences in how Ngb was expressed in response to severe ischemia versus milder ischemia or in the brain volume that underwent the ischemic insult. In summary, Ngb expression seems to depend on the species, region of ischemia, duration of the insult, and the biophysiological mechanism involved.

6.4 SPECIFIC AIM 4: DIFFERENCE IN FUNCTIONAL OUTCOMES OF PATIENTS WITH AND WITHOUT NGB VARAINTS (PRSENT/ABSENT) OVER TIME

We found with univariate analysis that there was a significant association between SNP1 variants and the GOS scores at months 3, 6, 12, and 24, but not for SNP2. Specifically, patients with SNP1 Vabsent had better outcomes on the GOS at all time points, as demonstrated in Figure 17. It seems that the difference was most dramatic at the 3-month time point, with a 17% difference in the percentage of patients classified as Vpresent or Vabsent. This percentage difference was maintained at approximately 20% over the next three time points. Thus, there does not seem to be additional improvement over time associated with the Ngb genotypes.

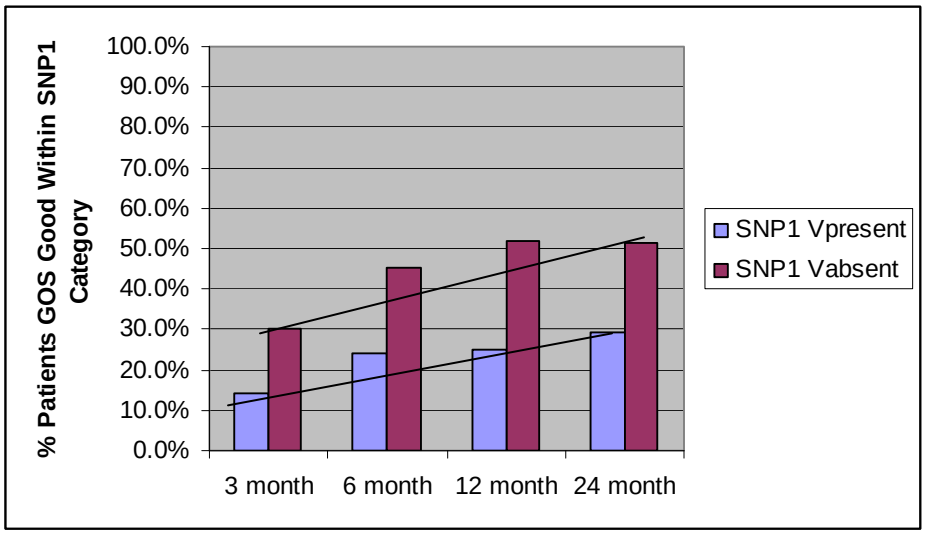


Figure 17. Relationship between SNP1 Variants and Good Outcome by GOS over Time

We found a similar relationship over time between SNP1 variants and outcomes assessed by the DRS (noting that lower DRS score is associated with better outcome [Figure 18]).

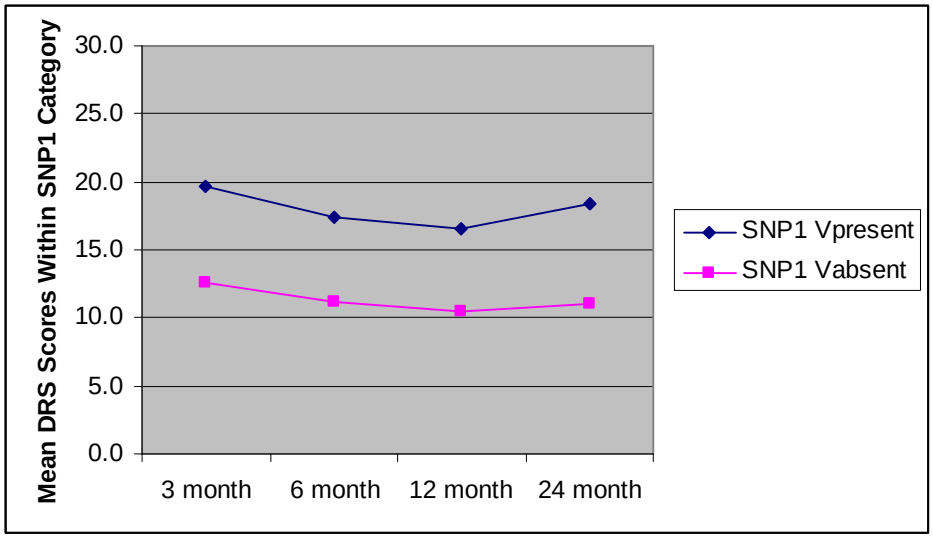


Figure 18. Relationship between SNP1 Variants and Good Outcome by DRS over Time

We found no relationship between SNP1 variants and outcome over time as assessed with the NRS (Figure 19); however, the number of patients with this outcome measurement available was small, which calls the strength of this result into question.

With logistic regression analyses, we found that being SNP1 Vabsent predicted a better outcome in some of our models, although its predictive strength was mitigated to some degree when we controlled for the severity of injury. This finding could be explained in several ways. One possible explanation is that the functional outcome was more likely to be a result of the severity of the initial injury than due to the availability of Ngb to protect against the insult. On the other hand, it is possible that Ngb and severity of injury are so closely correlated that it is difficult to separate the effects of these variables from one another. A third explanation is that Ngb may have been able to exert a protective effect while the patients were still in the field that could not be detected by the assessments conducted once the patients were admitted to the hospital.

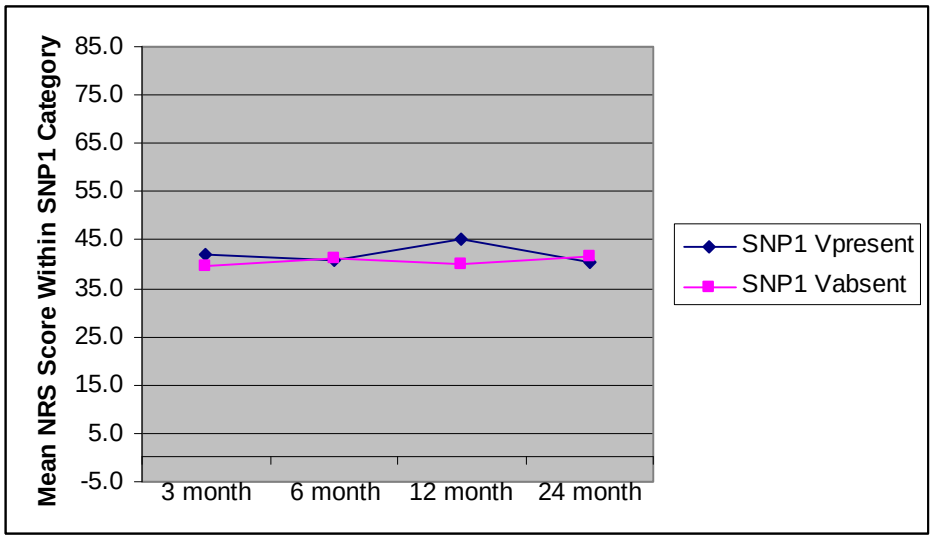


Figure 19. Relationship between SNP1 Variants and Good Outcomes by NRS over Time

We found no literature that described the impact of Ngb variants on functional outcomes in patients with TBI. We did find two studies conducted using animal models which examined the role of Ngb in functional outcomes after experimental stroke. Sun, Jim, Peel, Mao, Xie, and Greenberg (2003) manipulated Sprague-Dawley to either have reduced Ngb expression by administering Ngb anti-sense ODN or increased Ngb expression by intracerebral administration of an Ngb-expressing AVV vector. They subsequently measured functional outcome according to a neurologic grading scale that assessed motor, sensory, and reflex function, with higher scores indicating more severe impairment. They found that the groups of rats administered the Ngb antisense had worse neurological deficits than the rats administered the AVV ($p < 0.05$).

Wang et al (2008) compared Ngb overexpressing transgenic mice (Ngb-Tg) to wild typed controls after transient focal ischemia. They measured sensorimotor function in the mice in four ways at 1, 3, 7, and 14 days after the onset of ischemia: neurological score, rotorod test, hanging wire, and foot fault tests. They found significant deficits for all tests with the poorest scores between 1 and 7 days after the insult, but behavioral outcome was close to pre-injury baseline

values on all tests at 14 days. They did not find significant differences in sensorimotor function between their Ngb-Tg mice and the wild typed mice despite the fact that, as previously described, the Ngb-Tg mice had smaller infarct volumes. Based on these findings, they hypothesized that a) 60 min of MCAo might not be long enough to cause severe sensorimotor deficits, or b) the number of subjects might have been too small to detect between-group differences on this outcome variable.

Thus, our finding of improved outcomes after TBI based on the genetic Ngb variant in patients has some support in animal models of brain injury, but to our knowledge our findings are the first to describe a relationship between functional outcomes and Ngb in humans.

6.5 STATEMENT OF IMPORTANT FINDINGS AND SIGNIFICANCE TO NURSING

In summary, we observed that genetic variants of the Ngb genes were related to functional outcome; specifically, the presence of the Ngb variant in SNP1 was related to worse functional outcome. Although this finding was somewhat attenuated when the severity of injury, as measured by the GCS, and older age were taken into account, the finding suggests that the Ngb gene affects recovery as well as the severity of the injury on admission. This information is important to clinical nurses for several reasons: First, these results provide nurses with more insight into the physiological mechanisms that help to protect the brain from injury. Second, clinical nurses may be better able to understand and describe why patients with the same severity of injury can have a better or worse outcome by understanding the role of Ngb in these processes and that individuals may have different responses to an environmental stimulus because of a

genetic predisposition. Furthermore, such information is important to researchers because this is the first time that genetic variants in Ngb have been implicated in the severity of injury and functional outcome in humans. Therefore, these data will be extremely valuable for providing the framework and justification for further research aimed at describing the role that Ngb plays in protecting individuals from secondary brain injury and developing mechanisms to utilize Ngb treatment strategies.

6.6 LIMITATIONS OF THE STUDY

There are several limitations to our study. Because we found no information in the literature related to Ngb variants and TBI in humans, we were unable to compare our findings to others for validation. The number of non-Caucasians in our sample was very small (N = 12), and it would be interesting to have a large enough sample of non-Caucasian patients to see how the results of other races (analyzed separately) would compare to our sample of Caucasian patients. Therefore, our results are generalizable only to Caucasians. In addition, we were unable to measure the protein itself due to financial constraints. Perhaps direct protein measurement would have given different results. Our most important limitation was that we were required to use the GCS as our metric for the severity of TBI. GCS scores, however, are not a direct measure of cerebral blood flow and, therefore, cerebral hypoxia or ischemia. Having a more direct and reliable measure of cerebral ischemia would increase the validity and reliability of our findings and would enable us to more accurately examine the relationship between Ngb and severity of injury. Because the animal model studies indicated that Ngb may be expressed differently in focal ischemia versus global ischemia, access to information regarding the location of ischemia and its severity in our

sample of patients would have enabled us to better understand and explain the findings. Also, in this study, we measured only Ngb genetic variants and did not directly measure or detect gene expression (on/off) on mRNA or protein functions.

6.7 FUTURE DIRECTIONS FOR RESEARCH

There are several potential research plans which could evolve from this pilot study. First, I wish to examine the same SNPs in a larger sample of patients with TBI with a hypoxic-ischemic insult for which the results of Xenon blood flow studies, which directly measures cerebral ischemia, are available. Second, I would like to examine the same genetic variants in different patient populations with cerebral ischemia, such as stroke, subarachnoid hemorrhage, or brain tumor. Furthermore, it would be helpful to have more information about copy number variants in the exact gene region which corresponds to Ngb on chromosome14 in order to investigate more fully its role in possibly protecting the brain against hypoxic and ischemic conditions. Also, we examined the impact of the SNPs individually, but studying haplotypes may be more helpful. Additionally, these findings can serve to justify the pursuit of funding that would support subsequent research studies that utilize technologies that are more sensitive for measuring the Ngb protein itself, such as mass spectrometry, which would more directly examine the relationship between the presence of Ngb proteins and outcomes. Lastly, it would be interesting to examine Ngb gene expression (m RNA) in patients with different severities of TBI (for instance, mild, moderate, and severe TBI) or investigate the potential for functional SNPs in the region tagged by SNP1.

APPENDIX A

CLINICAL CARE PROTOCOL

UPMC
University of Pittsburgh Medical Center

PHYSICIAN ORDER SET

AUTHORIZATION IS GIVEN TO THE PHARMACY TO DISPENSE AND TO THE NURSE TO ADMINISTER THE GENERIC OR CHEMICAL EQUIVALENT WHEN THE DRUG IS FILLED BY THE PHARMACY OF THE UPMC HEALTH SYSTEM HOSPITAL - UNLESS THE PRODUCT NAME IS CIRCLED

Severe Traumatic Brain Injury Admission Protocol -- Physician Order Set

Admit to(Unit): _____ Attending Physician: _____

Diagnosis: **Severe traumatic brain injury** Condition: _____

Allergies: _____

Check All Orders that Apply with a ☒ *& All Handwritten Orders Should be* BLOCK PRINTED *for Clarity*

Communication Orders

☒ Notify Neurosurgery on-call for any of the following:

- Core temperature > 38.3° C
- pCO_2 < 33 or > 37
- Intracranial Pressure (ICP) ≥ 25 mm Hg
- Serum Na^+ ≥ 155mEq/L
- Hct < 30%
- CPP < 60 mm Hg
- MAP < 65 or > 110 mm Hg

☒ Contact Critical Care Medicine for insertion of the following catheters:

- Alsius Cool-Line® (cooling CENTRAL line)
- Arterial line

Vital Signs

☒ Record vital signs, including ICP, MAP, CPP, Licox $PbtO_2$, brain temperature, local blood flow, Bispectral Index (BIS), end tidal CO_2 ($EtCO_2$), and rectal temperature every 1 hr x 5 days or until ICP monitoring discontinued

Patient Care

☒ Maintain head at neutral alignment, 30° head of bed elevation

☒ Maintain core temperature between 36.5° C. - 37.5° C, set Alsius Cool Gard® machine at 36.5° C

☒ External Ventricular Drainage (EVD) **open** at 10 cm above midbrain, close every 1 hour and record ICP reading following 5 minutes equilibration; notify Neurosurgery if ICP ≥ 25 mm Hg

☒ Ventilator Management: Maintain arterial pCO2 = 33-37; adjust respiratory rate by 2 breaths/minute hourly prn to achieve goal

☒ Maintain CPP > 60 mm Hg and MAP > 65 mm Hg, follow fluid management orders per **Severe TBI: Fluid Resuscitation Protocol**

☒ Maintain ICP < 20 mm Hg, follow ICP management per **Intracranial Hypertension Order Set**

IV Fluids

☒ 0.9% Sodium Chloride with KCl 20 mEq/L at 100 ml/hour ☒ IV carrier solutions in 0.9% Sodium Chloride solution

Medications

☒ Propofol 20 mcg/kg/minute - IV infusion, titrate prn per **Severe Traumatic Brain Injury Sedation Protocol**

☒ Fentanyl 50 mcg/hour IV infusion, may titrate prn, per **Severe Traumatic Brain Injury Sedation Protocol**

☒ Phenytoin (**Dilantin**) 1 gram IV loading dose, if not previously given

☒ Phenytoin (**Dilantin**) 100 mg IV every 8 hours, continue for ☐ 7 days ☐ 4 weeks ☐ 6 weeks

Next dose due (military time): _____

☒ Enoxaparin 30 mg subq q12 hrs. Start 48 hrs after admission, notify Neurosurgery prior to 1st dose being administered DO NOT SUBSTITUTE

☒ Acetaminophen 650 mg NG/PR every 4 hours prn - core temperature > 38.0° C

☒ Famotidine (**Pepcid**) 20 mg IV every 12 hours

☐ Ampicillin/sulbactam (**Unasyn**) 3 grams IV every 6 hrs X 16 doses. **If penicillin allergy, call CCM for alternative.**

_____ _____

(**BLOCK** Print Name) (Signature)

Date / Time: _____ Pager # _____

Additional Handwritten Orders Should be Placed at the End of this Order Set.

☐ Order Set Faxed to Pharmacy by: (name / time) _____ Unit: _____

0031-01-U Form ID: PUH-1241 Last Revision Date: 02/08/2007

UPMC
University of Pittsburgh Medical Center

PHYSICIAN ORDER SET

AUTHORIZATION IS GIVEN TO THE PHARMACY TO DISPENSE AND TO THE NURSE TO ADMINISTER THE GENERIC OR CHEMICAL EQUIVALENT WHEN THE DRUG IS FILLED BY THE PHARMACY OF THE UPMC HEALTH SYSTEM HOSPITAL - UNLESS THE PRODUCT NAME IS CIRCLED.

IMPRINT PATIENT IDENTIFICATION HERE

Severe Traumatic Brain Injury Admission Protocol -- Physician Order Set

Labs

- [X] Serum EtOH level upon admission, if not done in E.D.
- [X] Urine Toxicology Screen upon admission, if not done in E.D.
- [ ] Pregnancy test upon admission, if not done in E.D.
- [X] Serum sodium every 6 hours during ICP monitoring period
- [X] Arterial blood gas every 6 hours and one hour following ventilatory adjustment, during ICP monitoring period
- [X] Phenytoin (**Dilantin**) level just prior to 4th maintenance dose
- [X] Serum osmolality every 6 hours, if mannitol in use

Research Obtain the following after research consent obtained:

- [X] CSF sampling q 4 hours x 24 hours, then q 6 hours x 4 days and place in 4G freezer
- [X] Daily 7 am red top blood draw x 7 days post admission, and place in 4G refrigerator
- [X] Daily 7 pm EVD / CSF drainage bag change, and place in 4G freezer.

Radiology

- [ ] CT Scan of the head with perfusion the next morning following admission — Reason: Post-Severe Traumatic Brain Injury
- [ ] CT angiogram the next morning following admission — Reason: Post-Severe Traumatic Brain Injury

If contrast allergy, substitute a CT scan of the head, no contrast

Consult the following services for TBI evaluation:

- [X] Physical Therapy and Occupational Therapy
- [X] Social Services
- [X] Rehabilitation Medicine
- [X] Neurophysiology for SSEP and BSER on day 4 and day 11 of admission call pager 6225

Additional Orders Should be BLOCK PRINTED for Clarity	
The following abbreviations are disallowed: u (unit), MS and MSO4 (morphine), MgSO4 (magnesium sulfate), µg (microgram), QD (daily), QOD (every other day), IU, (International Units), TIW (three times weekly) and BT (bedtime).	
Other Orders	**Medication Orders**
Safe Prescribing Practices: Verify all orders by reading the order back to the prescriber. Do not use zeros following a decimal point. Use a zero before a decimal point. Order IV medications by dose per time (e.g., mg/hr). Order levothyroxine in "mcg" (not "mg") doses.	

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Additional Handwritten Orders Should be Placed at the End of this Order Set.

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Severe Traumatic Brain Injury: Intracranial Hypertension -- Physician Order Set

ICP Maintenance: (Normal Pressure < 20)

Step

1. **ICP > 20**
   a. Continuous CSF drainage via EVD at 10 cm above midbrain, per initial **Severe Traumatic Brain Injury Admission Protocol**
   b. Verify adequate sedation/analgesia per initial **Severe Traumatic Brain Injury Sedation Protocol**
   c. Verify Hct ≥ 30%
2. **ICP consistently ≥ 25, after step 1**
   If **closed** ICP reading is 25-29 mm Hg after 2 consecutive hourly readings, or > 30 mm Hg for 1 hourly reading, notify Neurosurgery and proceed with Steps 2a-b.
   a. Mannitol 0.5 gms/kg IV x1, repeat x1 if no response in ICP
      1. Check serum osmolality q 6 hours
      2. Hold Mannitol for serum osmolality > 315 mOsm/kg
   b. **STAT** CT head scan to rule out mass lesion
3. **ICP consistently ≥ 25 after step 2**
   Obtain orders from Neurosurgery for proceeding with steps 3a-b
   a. Discontinue maintenance fluid and begin 3% hypertonic sodium chloride with KCl 20 mEq/L at 1 ml/kg/hr
      1. Serum Na^+ Goal 150-155 mEq
      2. If serum Na+ > 155 mEq, change to 0.9% sodium chloride with KCl 20 mEq/L at 100 ml/hr
   b. Decrease arterial pCO_2 (to Goal of 29-31)
      1. Increase ventilatory rate to reach pCO_2 Goal
      2. Once ICP < 25 mm Hg, notify Neurosurgery for additional ventilatory adjustments

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SEVERE TBI ORDERS
ICP MAINTENANCE PROTOCOL

Protocol Goals: The main goals of this protocol are to provide a standardized approach to ICP management in patients with severe TBI, and to provide the nurse with guidance to the next level of control for elevated intracranial pressure.

Protocol Triggers: The patient will be eligible for ICP management by protocol if they develop intracranial hypertension (ICP > 20 mmHg). ICP reading must be obtained in a 'closed to the head' stopcock position, after a 5 minute equilibration period.

Protocol Details:

1. The External Ventricular Drainage (EVD) system is to be maintained open at 10 cm above midbrain, for continuous CSF drainage. Every hour, close EVD stopcock to the head and record ICP reading following a 5 minute equilibration. Mark all other ICP readings as 'open' in the Emtek system.

2. If closed ICP reading is > 20 mm Hg verify adequate sedation/analgesia per **Severe Traumatic Brain Injury Sedation Protocol**. Consider neuromuscular blockade if increased ICP is associated with dyscoordinated breathing, posturing, excess motor activity or shivering unresponsive to sedation protocol limits. Notify Neurosurgery prior to vecuronium IV bolus (0.1 mg/kg q 45 minutes).

3. If closed ICP reading is 25-29 mm Hg after 2 consecutive hourly readings, or > 30 mm Hg for 1 hourly reading, notify Neurosurgery for escalation to 3% hypertonic saline, or hyperventilation orders. Concurrently, order STAT CT scan of the head, without contrast to rule out mass lesion. In addition, administer mannitol 0.5 gms/kg IV x 1. Repeat mannitol administration if no response in ICP.

4. Pentobarbital administration will only be a consideration after all other means of ICP control have been trialed, i.e. paralytics, osmotic therapy,, hyperventilation, hypothermia, decompressive craniectomy. Attending Neurosurgeon approval required.

Physician Notifications: Neurosurgery is to be notified prior to any escalation in protocol due to increased ICP, i.e. administration of paralytics, CT scan, mannitol administration. Additional orders must be obtained from Neurosurgery prior to hypertonic saline administration, hyperventilation and pentobarbital administration.

CSF Sampling:

1. The External Ventricular Drainage (EVD) system should be closed to the head prior to sampling (see Procedural Manual). Betadine scrub port for 3 minutes and follow sterile procedure.
2. Sampling is scheduled q 4 hours for the first 24 hours, and then every 6 hours for the next 4 days, unless EVD is discontinued.
3. Obtain 3 ml, if possible, from the tubing closest to the head, and place in red top glass tube.
4. Date and time stamp sample and place in a biohazard bag
5. Place in 4G freezer immediately following collection for lab pick-up .

This page is NOT a permanent part of the medical record.

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Severe Traumatic Brain Injury: Fluid Resuscitation Protocol -- Physician Order Set

Fluid Resuscitation Protocol Guidelines are on following page

Physician Notifications

- ☒ Any stops or interruptions in protocol because of adverse effects of fluid administration
- ☒ Any time vasopressor support is initiated with levophed
- ☒ Failure to achieve physiological endpoints within the constraints of the protocol

Orders to be implemented if hypotension or low cerebral perfusion pressure occurs

HYPOTENSION (MAP < 65 mmHg)					
	CVP	Challenge Volume	Fluid	Frequency	Maximum Volume
☒	< 5	1000 ml	Colloid	Bolus	1000 ml
			Sodium Chloride	q 30 min	2000 ml
☒	5-10	500 ml	Colloid	q 15 min	1000 ml
			Sodium Chloride	q 15 min	1000 ml
☒	11-12	250 ml	Colloid	q 10 min	500 ml
			Sodium Chloride	q 10 min	500 ml

LOW CEREBRAL PERFUSION PRESSURE (CPP < 60 mmHg)					
	CVP	Challenge Volume	Fluid	Frequency	Maximum Volume
☒	< 5	1000 ml	Sodium Chloride	q 60 min	2000 ml
☒	5-10	500 ml	Sodium Chloride	q 60 min	1000 ml
☒	11-12	No Fluid Challenge, Norepinephrine (**Levophed**) if MAP < 100 mmHg			

Fluid Challenge and Vasopressor administration details

- ☒ **5% albumin IV** 1000 ml maximum - administer only during first 24 hours after ICU admission.
- ☒ **Hetastarch in balanced salt solution (Hextend) IV** 1000 ml maximum/day, start > 24 hours after ICU admission
- ☒ Norepinephrine (**Levophed**) IV infusion at 0.1 mcg/kg/min for hypotension or low CPP unresponsive to 1 liter fluid challenge. *(CPP < 60 mmHg , CVP > 10 mmHg and MAP < 100 mmHg)* Titrate to MAP 65-70 mmHg or CPP > 60 mmHg.

- ☒ Maximum colloid permitted is 1 liter every 24 hours.
- ☒ For all colloid challenges, terminate challenge if BP endpoint achieved prior to receiving full challenge volume.
- ☒ If patient requires vasopressor to achieve physiological endpoints, continue volume challenges up to protocol volume limits and attempt weaning from vasopressor.

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Severe Traumatic Brain Injury: Sedation Protocol -- Physician Order Set

Analgesia- Fentanyl

☒ Begin fentanyl **IV** infusion at 50 micrograms/hour upon admission to the ICU.

☒ **PRN dose and Indication (this is in addition to the ongoing Fentanyl infusion)**: Fentanyl 100 micrograms IV q 1 hour prn for ventilator dysynchrony, hyperventilation, posturing, excess motor activity, shivering, episodic hypertension (MAP > 110 mmHg) or episodic tachycardia (HR > 110) or any clinical indicator suggesting unrelieved pain.

☒ Increase fentanyl **IV** infusion by 50 micrograms/hour every 4 hours to a maximum **IV** infusion rate of 200 micrograms per hour if prn Fentanyl boluses > 200 micrograms in preceding 4 hour period.

☒ Reduce fentanyl **IV** infusion for neuro exam every morning at **6am** to 50 micrograms/hr unless ICP > 25 mmHg; there is an indication for a prn bolus of fentanyl; or the patient is receiving neuromuscular blockers. Maintain **IV** infusion at 50 micrograms/hr unless additional bolus doses are indicated.

Sedation- Propofol

☒ Begin propofol **IV** infusion at 20 micrograms/kg/minute upon admission to the ICU.

☒ **PRN Dose and Indications**: Propofol bolus 0.5 mg/kg IV q 1 hour prn for posturing, restlessness, excess motor activity, shivering, episodic hypertension, episodic tachycardia resistant to fentanyl boluses.

☒ **PRN Dose for Increased ICP**: Propofol bolus 0.25 mg/kg IV q 1 hour prn for ICP elevation ≥ 25 mmHg

☒ Increase propofol **IV** infusion by 10 micrograms/kg/min q 1 hour following any prn propofol bolus to a maximum of 100 micrograms/kg/min.

☒ Stop propofol infusion at **7am** every day for neuro exam unless ICP ≥ 25, there is an indication for a prn bolus, or the patient is receiving neuromuscular blockers. Observe patient off of propofol and restart infusion at 50% of prior dose if there remains an indication to continue propofol.

Neuromuscular Blockade - Vecuronium

☒ Vecuronium 0.1 mg/kg **IV** bolus q 45 minutes prn dyscoordinated breathing unresponsive to sedation, intracranial hypertension (ICP > 25 mmHg) associated with posturing, excess motor activity, or shivering unresponsive to 100 micrograms of fentanyl and 0.50 mg/kg **IV** bolus of propofol.

Other

☒ Hold propofol for hypotension (MAP < 65 mmHg) or vasopressor requirements

☒ Obtain triglyceride level daily for all patients on propofol dose > 50 micrograms/kg/min for > 24 hours

☒ Notify physician for uncontrollable motor activity, ventilator dysynchrony and the need for vecuronium

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APPENDIX B

DISABILITY RATING SCALE (DRS)

Patient Name: _____ Date of Rating:_____

Name of Person Completing Form: _____

DISABILITY RATING SCALE:

A. EYE OPENING:

- ☐ (0) Spontaneous
- ☐ (1) To Speech
- ☐ (2) To Pain
- ☐ (3) None

0-SPONTANEOUS: eyes open with sleep/wake rhythms indicating active arousal mechanisms, does not assume awareness.
1-TO SPEECH AND/OR SENSORY STIMULATION: a response to any verbal approach, whether spoken or shouted, not necessarily the command to open the eyes. Also, response to touch, mild pressure.
2-TO PAIN: tested by a painful stimulus.
3-NONE: no eye opening even to painful stimulation.

B. COMMUNICATION ABILITY:

- ☐ (0) Oriented
- ☐ (1) Confused
- ☐ (2) Inappropriate
- ☐ (3) Incomprehensible
- ☐ (4) None

0-ORIENTED: implies awareness of self and the environment. Patient able to tell you a) who he is; b) where he is; c) why he is there; d) year; e) season; f) month; g) day; h) time of day.
1-CONFUSED: attention can be held and patient responds to questions but responses are delayed and/or indicate varying degrees of disorientation and confusion.
2-INAPPROPRIATE: intelligible articulation but speech is used only in an exclamatory or random way (such as shouting and swearing); no sustained communication exchange is possible.
3-INCOMPREHENSIBLE: moaning, groaning or sounds without recognizable words, no consistent communication signs.
4-NONE: no sounds or communications signs from patient.

C. MOTOR RESPONSE:

- ☐ (0) Obeying
- ☐ (1) Localizing
- ☐ (2) Withdrawing
- ☐ (3) Flexing
- ☐ (4) Extending
- ☐ (5) None

0-OBEYING: obeying command to move finger on best side. If no response or not suitable try another command such as "move lips," "blink eyes," etc. Do not include grasp or other reflex responses.
1-LOCALIZING: a painful stimulus at more than one site causes limb to move (even slightly) in an attempt to remove it. It is a deliberate motor act to move away from or remove the source of noxious stimulation. If there is doubt as to whether withdrawal or localization has occurred after 3 or 4 painful stimulations, rate as localization.
2-WITHDRAWING: any generalized movement away from a noxious stimulus that is more than a simple reflex response
3-FLEXING: painful stimulation results in either flexion at the elbow, rapid withdrawal with abduction of the shoulder or a slow withdrawal with adduction of the shoulder. If there is confusion between flexing and withdrawing, then use pinprick on hands.
4-EXTENDING: painful stimulation results in extension of the limb.
5-NONE: no response can be elicited. Usually associated with hypotonia. Exclude spinal transection as an explanation of lack of response; be satisfied that an adequate stimulus has been applied.

D.FEEDING (COGNITIVE ABILITY ONLY)

- ☐ (0.0) Complete
- ☐ (0.5) Btw.Compl/partial
- ☐ (1.0) Partial
- ☐ (1.5) Btw.partial / minimal
- ☐ (2.0) Minimal
- ☐ (2.5) Btw.min/none
- ☐ (3.0) None

Does the patient show awareness of how and when to perform this activity? Ignore motor disabilities that interfere with carrying out this function. (This is rated under Level of Functioning described below.)
0-COMPLETE: continuously shows awareness that he knows how to feed and can convey unambiguous information that he knows when this activity should occur.
1-PARTIAL: intermittently shows awareness that he knows how to feed and/or can intermittently convey reasonably clearly information that he knows when the activity should occur.
2-MINIMAL: shows questionable or infrequent awareness that he knows in a primitive way how to feed and/or shows infrequently by certain signs, sounds, or activities that he is vaguely aware when the activity should occur.
3-NONE: shows virtually no awareness at any time that he knows how to feed and cannot convey information by signs, sounds, or activity that he knows when the activity should occur.

E.TOILETING (COGNITIVE ABILITY ONLY)

- ☐ (0.0) Complete
- ☐ (0.5) Btw.Complete/partial
- ☐ (1.0) Partial
- ☐ (1.5) Btw.partial / minimal
- ☐ (2.0) Minimal
- ☐ (2.5) Btw.minimal / none
- ☐ (3.0) None

Does the patient show awareness of how and when to perform this activity? Ignore motor disabilities that interfere with carrying out this function. (This is rated under Level of Functioning described below.) Rate best response for toileting based on bowel and bladder behavior
0-COMPLETE: continuously shows awareness that he knows how to toilet and can convey unambiguous information that he knows when this activity should occur.
1-PARTIAL: intermittently shows awareness that he knows how to toilet and/or can intermittently convey reasonably clearly information that he knows when the activity should occur.
2-MINIMAL: shows questionable or infrequent awareness that he knows in a primitive way how to toilet and/or shows infrequently by certain signs, sounds, or activities that he is vaguely aware when the activity should occur.
3-NONE: shows virtually no awareness at any time that he knows how to toilet and cannot convey information by signs, sounds, or activity that he knows when the activity should occur.

APPENDIX C

NEUROHEBAVIROAL RATING SCALE (NRS)

Neurobehavioral Rating Scale

DIRECTIONS: Place an X in the appropriate box to represent level of severity of each symptom

	Not Present	Very Mild	Mild	Moderate	Mod-Severe	Severe	Extremely Severe
1. INATTENTION / REDUCED ALERTNESS—fails to sustain attention, easily distracted, fails to notice aspects of environment, difficulty directing attention, decreased alertness.	❑	❑	❑	❑	❑	❑	❑
2. SOMATIC CONCERN—volunteers complaints or elaborates about somatic symptoms (e.g., headache, dizziness, blurred vision), and about physical health in general.	❑	❑	❑	❑	❑	❑	❑
3. DISORIENTATION—confusion or lack of proper association for person, place, or time.	❑	❑	❑	❑	❑	❑	❑
4. ANXIETY—worry, fear, overconcern for present or future.	❑	❑	❑	❑	❑	❑	❑
5. EXPRESSIVE DEFICIT—word-finding disturbance, anomia, pauses in speech, effortful and agrammatic speech, circumlocution.	❑	❑	❑	❑	❑	❑	❑
6. EMOTIONAL WITHDRAWAL—lack of spontaneous interaction, isolation, deficiency in relating to others.	❑	❑	❑	❑	❑	❑	❑
7. CONCEPTUAL DISORGANIZATION— thought processes confused, disconnected, disorganized, disrupted; tangential social communication; perseverative.	❑	❑	❑	❑	❑	❑	❑
8. DISINHIBITION—socially inappropriate comments and / or actions, including aggressive / sexual content, or inappropriate to the situation, outbursts of temper.	❑	❑	❑	❑	❑	❑	❑
9. GUILT FEELINGS—self-blame, shame, remorse for past behavior.	❑	❑	❑	❑	❑	❑	❑
10. MEMORY DEFICIT—difficulty learning new information, rapidly forgets recent events, although immediate recall (forward digit span) may be intact.	❑	❑	❑	❑	❑	❑	❑
11. AGITATION—motor manifestations of overactivation (e.g., kicking, arm flailing, picking, roaming, restlessness, talkativeness.)	❑	❑	❑	❑	❑	❑	❑
12. INACCURATE INSIGHT AND SELF-APPRAISAL—poor insight, exaggerated self-opinion, overrates level of ability and underrates personality change in comparison with evaluation by clinicians and family.	❑	❑	❑	❑	❑	❑	❑
13. DEPRESSIVE MOOD—sorrow, sadness, despondency, pessimism.	❑	❑	❑	❑	❑	❑	❑
14. HOSTILITY / UNCOOPERATIVENESS—animosity, irritability, belligerence, disdain for others, defiance of authority.	❑	❑	❑	❑	❑	❑	❑
15. DECREASED INITIATIVE / MOTIVATION—lacks normal initiative in work or leisure, fails to persist in tasks, is reluctant to accept new challenges.	❑	❑	❑	❑	❑	❑	❑
16. SUSPICIOUSNESS—mistrust, belief that others harbor malicious or discriminatory intent.	❑	❑	❑	❑	❑	❑	❑
17. FATIGABILITY—rapidly fatigues on challenging cognitive tasks or complex activities, lethargic.	❑	❑	❑	❑	❑	❑	❑
18. HALLUCINATORY BEHAVIOR—perceptions without normal external stimulus correspondence.	❑	❑	❑	❑	❑	❑	❑
19. MOTOR RETARDATION—slowed movements or speech (excluding primary weakness).	❑	❑	❑	❑	❑	❑	❑
20. UNUSUAL THOUGHT CONTENT—unusual, odd, strange, bizarre thought content.	❑	❑	❑	❑	❑	❑	❑
21. BLUNTED AFFECT—reduced emotional tone, reduction in normal intensity of feelings, flatness.	❑	❑	❑	❑	❑	❑	❑
22. EXCITEMENT—heightened emotional tone, increased reactivity.	❑	❑	❑	❑	❑	❑	❑
23. POOR PLANNING—unrealistic goals, poorly formulated plans for the future, disregards prerequisites (e.g., training), fails to take disability into account.	❑	❑	❑	❑	❑	❑	❑
24. LIABILITY OF MOOD—sudden change in mood which is disproportionate to the situation.	❑	❑	❑	❑	❑	❑	❑
25. TENSION—postural and facial expression of heightened tension, without the necessity of excessive activity involving the limbs or trunk.	❑	❑	❑	❑	❑	❑	❑
26. COMPREHENSION DEFICIT—difficulty in understanding oral instructions on single or multiusage commands.	❑	❑	❑	❑	❑	❑	❑
27. SPEECH ARTICULATION DEFECT—misarticulation, slurring or substitution of sounds which affect intelligibility (rating is independent of linguistic content).	❑	❑	❑	❑	❑	❑	❑

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APPENDIX D

DNA EXTRACTION PROTOCOL

D.1 CSF EXTRACTION WITH QIAMP MIDI DNA KITS

1. Pipette 200 ul of protease into 15 ml conical tube.
2. Add 2ml of CSF and mix briefly. Make sure to mix CSF well before adding to conical tube (15ml).
3. Add 2.4 ml Buffer AL and mix thoroughly by vortexing at least 3x 5sec each time.
4. Incubate at 70°C for 10 minutes.
5. Add 2ml 96-100% Ethanol (EtOH) and mix by vortexing.
6. Transfer half solution to Midi column resting in 15 ml conical tube. DO NOT SPILL AND DO NOT GET ON TRIM. Close cap and centrifuge at 3000 rpm for 3 minutes.
7. Remove column from tube and discard filtrate. Place column back into tube and add rest of solution to column. Spin again at 3000 rpm for 3 minutes.
8. Remove column and discard filtrate. Place column back into tube.
9. Add 2 ml Buffer AW1 to column. Close cap and centrifuge at 5000 rpm for 15 minutes.
10. Place column in clean 15 ml conical and discard old conical with wash buffers.
11. Place column in clean 15 ml conical and discard old conical with wash buffers.
12. Add 300 ul of Buffer AE to column. Close cap and incubate at room temp for 5 minutes. The centrifuge at 5000 rpm for 5 minutes.
13. Reload the 300 ul you just spun down into column. Incubate 5 minutes and spin for 5 minutes.
14. Load 300 ul of fresh AE Buffer into column. Incubate 5 minutes and spin 5 minutes.

15. Only after checking labels, combine DNA extractions into 1.5 ml samples tube and label appropriately.

Notes: CSF is live so work under hood until EtOH is added. EtOH will kill pathogens then work can be done on bench. Remember to label 15 ml conical and columns. This will avoid cross contamination. Preheat incubator before starting extraction. It takes a while to get up to 70°C also make sure to remove anything in the incubator prior to turning the temp up!!
Use filter tips and appropriate pipettors.
Cleanup: Waste down the sink. Tubes in biohazard. Clean area up appropriately.

D.2 SOP FOR DNA EXTRACTION

Note: Run 16 samples at one time.
Disposal: All used tubes and anything contaminated with specimen must be placed in biohazardous waste to be autoclaved. EtOH is to be disposed of in approved container or an empty ethanol bottle. Non-contaminated pipettes can be disposed of in broken glass containers.

1. Take 50 ml conical tubes with pellets out of -20°C freezer to thaw
2. Place 3 ml lysis solution for DNA extraction in each tube. Vortex and using transfer pipette, transfer contents to labeled 15 ml conical tube.
3. Add 200uL of 10% SDS (already made)

   Add 500uL of proteinase K solution (must be made fresh each time)

Proteinase K solution:	10% SDS	900 uL
	0.5 M EDTA	36 uL
	Proteinase K	18 mg
	Sterile H_2O	8.1 mL
	Total	9.0 m L (enough for 18 samples)

4. Parafilm tubes shut to seal. Place in 37°C rotating oven overnight to digest.
5. Remove tubes from the oven. Add 1 ml 6M (saturated) NaCl/tube. Shake about 15 seconds until foamy.
6. Centrifuge 15 minutes at 2500 rpm.
7. Transfer supernatant to labeled 15 ml conical tubes. CHECK LABELS!!
8. Add 2x the volume of absolute EtOH and invert until the DNA precipitates out of the solution. It will look white and stringy.
9. Remove DNA using sterile loops and place in labeled flip tops.
10. Add 70% EtOH to cover the DNA and microfuge for 10 minutes at the 14 settings.
11. Dump or pipette off the EtOH. Then place in 37°C oven until EtOH evaporates off.
12. Add 1 ml of 1x TE buffer to each tube. Place in incubator to dissolve DNA into buffer.

Approved by: _____ Date: _____

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