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TITLE: Biomarkers Prognostic for Elevated Intracranial Pressure (DM090902)

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<b>14. ABSTRACT</b> Traumatic brain injury (TBI) is a major health problem, both for the military and civilian populations. Delayed brain swelling (typically occurring 2-3 days after the initial trauma) is a prominent secondary pathology that greatly contributes to poor outcome and death. Since the brain is confined within the skull, swelling increases intracranial pressure (ICP) that can decrease cerebral blood flow and shift brain tissue from its normal location. These events can cause further brain damage and, if untreated, can result in death. Not all persons with TBI will develop elevated ICP, even though their injury severities are similar. At present, there is no simple and rapid test (e.g. blood test) available to identify those patients at risk for developing increased ICP. Using blood samples from a group of severe TBI patients, we have found that the copper-binding protein ceruloplasmin and copper decrease in patients who will subsequently develop elevated ICP. These changes occur within the first 24 hours of injury making them potential markers that can be used to diagnose this condition. The goal of the proposed study is to validate these initial findings and determine the sensitivity and specificity of these makers to identify TBI patients at risk for elevated ICP.					
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## 1. INTRODUCTION:

High intracranial pressure (ICP) is a serious secondary pathology after traumatic brain injury (TBI) that can decrease cerebral perfusion to the injured brain, cause brain tissue herniation, and further brain damage. As elevated ICP is associated with increased morbidity and mortality, determination of which patients will develop this devastating condition is critical to ensure proper intervention. Unfortunately, there are no clinically proven tests for predicting which TBI patients will develop high ICP. Using a LC-MS/MS proteomic approach, we have identified candidate serum biomarkers whose levels are markedly altered in people with TBI, with the goal of identifying those markers which are predictive of elevated ICP. Ceruloplasmin, also called iron(II):oxygen oxidoreductase, is the major copper carrier protein in the blood and plays an important role in copper and iron metabolism. Our initial studies using plasma from healthy volunteers, TBI patients with  $ICP \leq 20$  mm Hg (the threshold for intervention), and TBI patients with  $ICP \geq 25$  mm Hg indicate that plasma ceruloplasmin is a very good biomarker (AUC = 0.86) for predicting the occurrence of high ICP in people with TBI. Of particular interest is our observation that in patients who develop high ICP, there is a significant reduction in plasma ceruloplasmin and total copper levels within the first 24hr of injury, hours-to-days before ICP increased above 25 mm Hg. The purpose of this project was to confirm these preliminary findings using a larger number of study subjects, and to determine the values that can be used to prognose/diagnose elevated ICP.

**Aim 1.** To determine the cut-off concentrations of ceruloplasmin and copper that can be used to classify TBI patients on their subsequent ICP status.

**Aim 2.** To verify the diagnostic accuracy of ceruloplasmin and copper in identifying TBI patients at risk for developing elevated ICP using blinded samples.

**Aim 3.** To evaluate the influence of temperature on the biomarker assays, and to determine if whole blood can be used.

**2. KEYWORDS:** Traumatic brain injury, biomarker, plasma, copper, ceruloplasmin, intracranial pressure

## 3. ACCOMPLISHMENTS:

### 3a) Major goals of the project:

**Task 1 (0-6 months).** Acquire all necessary approvals from the Institutional Review Boards of the University of Texas Medical School and Memorial Hermann Hospital, as well as from the DOD prior to initiating sample collection.

**Status: Completed**

**Task 2 (7-33 months).** Patient enrollment. All study subjects who meet the inclusion criteria of this study will be included in this project. The results obtained from these studies will not be used to alter their treatment, nor will the results of the measures be conveyed to the attending physician. Blood will be withdrawn and processed for the analysis of plasma ceruloplasmin and copper levels. Processing will take place at the Memorial Hermann Hospital Laboratory Services or the University of Texas Health Science Center at Houston. Laboratory samples will be coded with a barcode, aliquoted and frozen.

**Status: Completed**

**Task 3 (7-24 months).** Ceruloplasmin and copper assays will be carried out for patients whose group designations are known in order to determine cut-off values that can be used to identify patients at risk for developing elevated ICP.

**Status: Completed**

**Task 4 (18-33 months).** Using the cut-off values obtained from task 3, we will test the diagnostic accuracy of plasma ceruloplasmin and copper for their ability to predict which TBI patients will have a subsequent elevation in ICP.

Status: Completed

**Task 5.** Following the completion of Aims 1 and 2, the accuracy of the ceruloplasmin and copper tests when carried out at different temperatures will be assessed, as well as determining if whole blood can be used as the starting material.

Status: Not performed

### 3b) What was accomplished under these goals?

**Enrollment.** Over the course of this study, we screened almost 2200 patients with TBI to determine if they met study inclusion criteria. From these 2191 persons, 120 were enrolled in the study. The categorization of the screened individuals and their reasons for exclusion are documented in Table 1.

Table 1.

Enrolled	120	6%
Not expected to have ICP monitor	1176	54%
Multiple Reasons	301	14%
Outside of age range	224	10%
Not a traumatic brain injury	158	7%
Unsalvageable	139	6%
Could not obtain blood within $\leq 12$ hrs post injury	54	2%
Declined	3	0%
Withdrew	10	0%
Prisoner	5	0%
<b>Total Screened</b>	<b>2191</b>	<b>100%</b>

From the 120 enrolled patients, 56 could be classified as having elevated ICP ( $ICP \geq 25$ mmHg), 32 had no reported elevation in ICP ( $ICP \leq 20$ mmHg), and 13 had ICP values between 20mmHg and 25mmHg (Table 2). Nine subjects could not be easily classified due to monitor-related issues. The remaining 10 subjects were withdrawn from the study based on either an inability to obtain consent or a withdrawal of consent.

Table 2

Enrolled (n=110) (excluding those who withdrew)		
Male	91	83%
Female	19	17%
Age (average $\pm$ standard deviation, years)	37.5 $\pm$ 13.7	
Ethnicity		
Hispanic or Latino	40	36%
Not Hispanic or Latino	70	64%
Race		
White	96	87%
Black/African American	14	13%
ICP Groupings		
$ICP \geq 25$ mm Hg	56	51%
$ICP \leq 20$ mm Hg	32	29%
$>20, <25$	13	12%
Not available/unclear	9	8%
DRS (average $\pm$ standard deviation)	17.0 $\pm$ 11.0	

**Death Reports:** Twenty-six enrolled subjects have died since project initiation (21.6%). None of the deaths were considered related to this observational study. As per our IRB approval, deaths were reported at the time of our continuing review, and have also been detailed in our previous quarterly progress reports to the DoD.

***CC-TBI-001*** was a 38-year-old white female who was reportedly jogging and was struck by a car on 10/05/2011. On presentation to the ED she was normotensive but tachycardic and had a GCS of 5 with 4 mm, nonreactive pupils bilaterally. She was intubated in the ED. Head CT showed closed head injury with subdural hemorrhage, subarachnoid hemorrhage, and cerebral contusion, and skull base fracture with extension into the carotid canal. CTA of the neck confirmed that she had non-flow limiting dissection of the right internal carotid artery near the skull base and interval enlargement movement of the parenchymal hemorrhages in her cerebellar vermis and brainstem. Additionally, she had a left superior and inferior pubic ramus fracture and a left sacral fracture with a left pelvic hematoma and active extravasation, for which she was taken emergently to interventional radiology for embolization of the bilateral internal iliac arteries as well as the right internal iliac artery and right pudendal artery. She had L4 and L5 transverse process fractures and a mesenteric hematoma. Discussion with her next of kin described the grave prognosis of the patient. The decision was made that further care would have been futile given her neurological status and to provide comfort care. She was pronounced dead 1700 hours on 10/8/2011.

***CC-TBI-007*** was an 18 year old African American female who experienced an auto-pedestrian accident on 12/19/2011. She was a GCS of 4 in the field and intubated on the scene. She had a GCS 3T in the ED with pupils not equal and minimally reactive. Head CT showed multiple punctate contusions and trace intraventricular hemorrhage (IVH) consistent with diffuse axonal injury (DAI). She was requiring multiple pressors and continued to be unresponsive. Family meetings discussed her very poor prognosis and a decision to make the patient DNR was followed by the decision to provide comfort measures only(12/27/2011 at 13:30P). The patient was extubated at 14:52P, and shortly after passed with family at bedside.

***CC-TBI-008*** was a 57-year-old man who was riding his bike and was struck by a car. He presented to the ER with a GCS of 3 on 12/19/2011. He was intubated in the ER, he was found to have a right frontal bone fracture extending the skull base lesser sphenoid wing fracture, optic canal fracture, bilateral nasal bone fracture, a large subarachnoid hemorrhage, bilateral tentorial subdural hemorrhages, bilateral convexity subdural with midline shift as well as bifrontal contusions, right rib fractures of 6 and 7 ribs and a comminuted fracture of the pubic body and the pubic symphysis as well as right inferior pubic ramus fracture and an ileum fracture. He had progression of his injury on CT scan, but improvement of his neurologic exam, he was taken to the OR for an emergent craniectomy. He coded for 8 minutes before the craniectomy procedure with the craniectomy and was brought to the shock trauma ICU for monitoring and resuscitation. He had a very poor prognosis with little likelihood of return any significant neurologic function. After discussion with the family, the decision was made to withdraw life support measures. He was pronounced dead at 12/24/2011 12:19 a.m. The family was at the bedside.

***CC-TBI-009*** was a 50-year-old male who was involved in a motorcycle crash on 01/06/12, and suffered multiple injuries including a devastating head injury. Patient had IVH and SAH and hemorrhagic contusions s/p EVD by Neurosurgery. Patient also demonstrated chronic respiratory failure s/p tracheostomy and Dysphagia due to CVA s/p PEG tube for feeding access. During a family meeting with patient's mother, she stated explicitly that her son would not want to live in this condition and she understands that there is no chance at a functional recovery and that he would never be able to do the things that he enjoyed. On 1/27/12, family has decided to withdraw care and make patient comfort care only. Patient was transferred to Hospice on 1/30/12. Patient expired on 2/8/2012.

**CC-TBI-011** was a 29-year-old Hispanic male with no known past medical history. Patient was admitted on 1/27/2012 status post gunshot wound to the head with subsequent traumatic subdural, subarachnoid, intraventricular, intracranial hemorrhage and diffuse edema and multiple bone fragments. Patient underwent right hemicraniotomy for elevated intracranial pressure and tracheostomy for respiratory failure. Patient care transferred to palliative/hospice care after family meeting which took place on 2/7/2012. Patient expired on 2/9/2012 at 2:15 a.m.

**CC-TBI-015** was a 46-year-old male brought to MHH after a fall of approximately 30 feet with direct impact of head onto concrete. Patient was GCS 3 at the scene. He was intubated with a king tube in the field and brought to the ED. On initial presentation in the ED, he was GCS of 7T and found to have bifrontal, botemporal subdural hematoma, and pneumocephalus, diffuse subarachnoid hemorrhage, diffuse brain edema w/ sulci and brainstem effacement, small hemorrhagic bifrontal contusions, multiple skull and facial fractures. On arrival to Shock Trauma ICU, his GCS was 3 and he was on maximum dosages of levophed. EVD opening pressure was 25. Overnight, patient became bradycardic and hypotensive to the 60's diastolic requiring epi and initiation of vasopressin and epinephrine drips. He was also given calcium chloride and bicarbonate at this time. Patient was GCS 3T throughout his STICU stay and not overbreathing the ventilator. Time of death was determined to be 12:17 on 2/24/2012 based on cerebral blood flow scan.

**CC-TBI-018.** This patient was a 63-year-old male who was involved in a high-speed motorcycle collision without helmet who had a positive loss of consciousness per emergency medicine services. Patient was a GCS of 5 at the scene, and was hypoxic for approximately 20 to 30 minutes. On arrival at the ED, the patient was a GCS of 3 with pupils that were 3-mm and nonreactive. On head CT, it was noted that the patient had a left temporal convexity subdural hematoma measuring 1.1 cm with 2-mm left-to-right midline shift, extensive left bifrontal and left temporal parietal subarachnoid hemorrhage extending along the tentorium. Also, a left posterior parietal epidural hematoma measuring up to 8 mm, dissociated fracture, and local mass effect with minimal pneumocephalus were observed. No hydrocephalus was noted. The patient was loaded with Dilantin and an external ventricular drain was placed and the patient's opening pressure was noted to be 38. The patient was given mannitol. At that time, the patient was only GCS of 3 without corneal, gag or cough reflexes but was over-breathing the ventilator. Patient was started on normal saline to push the sodium up. On follow-up head CT, the patient was noted to have worsening subdural hematoma and was noted to have a poor prognosis. In discussions with the patient's family members it was decided to provide comfort care. After extubation, the patient went into cardiopulmonary arrest and died on 3/7/2012.

**CC-TBI-19** was a 50 year old male with ETOH use who fell at a gas station sustaining an acute on chronic SDH on 3/5/12 at 20:00. He was transferred from the ED to the OR where he had a right sided hemicraniectomy and evacuation of SDH. The patient's condition did not improve, and evaluation of EEG was negative for seizures. Neurosurgery met with family and given the poor likelihood of recovery, the patient was transferred to hospice care on 3/14/2012. The patient died on 3/16/2012 at 10:20.

**CC-TBI-023** was a 35 year old female unhelmeted passenger on a motorcycle who fell backwards off the motorcycle. She was found unresponsive and intubated on scene by EMS. Patient was seen by neurosurgery and trauma and based on exam and findings on head Ct, was felt to have a non-survivable brain injury. Patient was admitted to the ICU and monitored overnight. She was noted to have an improved exam, with posturing, therefore, a more aggressive approach to injuries was carried out. She was sent to IR for evaluation of her grade 3 liver laceration with active punctate extravasation and later had an exploratory-lap for concern of ischemic bowel, which was negative. Patient was a GCS 3, with elevated ICP. She required vasopressors to maintain MAP greater than 65 and CPP greater than 60 and had diabetes insipidus. Additionally, the patient had suffered a right forearm de-gloving injury. The patient was consistently GCS 3T and only with a minimal corneal reflex on the left eye, no other brainstem

reflexes. After a family meeting in which it was discussed that the patient had a non-survivable brain injury and no interventions could be done, the decision was made to provide comfort measures. LifeGift consented family for organ donation. On 4/2/2012 the patient expired at 18:23. The patient was then taken to OR for transplant surgery.

**CC-TBI-035** was a 45 year old male who was assaulted. Initial CT scan revealed multiple hemorrhages in midbrain and extra-axial. Neurosurgery placed a Camino bolt at bedside on 06/05/12 at 00:30 (opening pressure of 4). The bolt was removed and replaced with an EVD on 06/06/12 at 15:15. Continued ICP management with EVD in place through 6/10. On 6/14/12, a family meeting was held to discuss the patient's condition and that he would not improve beyond his current condition. The decision was made on 06/14/12 to continue with palliative care only. The ventilator was turned off after 9pm on 6/14/12 and the patient was pronounced dead at 02:43 on 06/15/12.

**CC-TBI-036** was a 38 year old male who was struck by a car on 6/12/12. Patient was GCS of 3 on scene. In CT, patient had transient drop of blood pressure to 85/50, then stabilized to 125/76. Neurosurgery placed a Camino bolt at bedside in ER (opening pressure of 9). He was admitted to the ICU in critical condition. Continued ICP management with bolt in place through 6/18. The bolt removed by neurosurgery on 6/18/12. On 6/21/12, the patient had an episode of bradycardia and hypotension to which his care team responded by administering Levophed for BP management. Afterward, the patient's pupils became fixed and dilated with no corneal or gag reflex. A stat CT scan showed evolving and additional hemorrhaging. He continued to be a GCS 3T off sedations. A cerebral blood flow study was ordered revealing absent cortical blood flow. Time of death was pronounced on 6/21/12 at 19:35. The patient remained in the ICU until 6/22/12 when he was taken to the OR for organ donation.

**CC-TBI-044** was a 63 year old male who was found unconscious on the highway with a head injury on 09/24/12. The patient was reported as a GCS 6 on the scene. The patient was brought to Memorial Hermann and found to have bilateral thalamic hemorrhages, bifrontal contusions, right parietal and left occipital contusions. Neurosurgery placed a Camino bolt at bedside in the ICU (opening pressure of 5). ICU care continued with the bolt in place through 09/26/12. The bolt was removed on 09/26/12. An EEG was performed on 09/28/12 which was markedly abnormal due moderate-to-severe diffuse slowing and suppression of cerebral activity. The patient was determined to have a poor prognosis due to the absence of significant reactivity to external stimulation. A family meeting was conducted with the patient's nephew over the phone who agreed to palliative care for the patient. The patient was transferred to hospice on 10/3/2012. The hospice reported the patient died on 10/5/2012.

**CC-TBI-045** was a 37 year old male who was hit by a motor vehicle on 09/29/12. The patient was reported to be a GCS 3 on the scene. The patient was brought to Memorial Hermann and found to have a right sided intracerebral hemorrhage and an intraventricular hemorrhage. Neurosurgery placed an external ventricular drain (EVD) in the ICU (opening pressure of 12). ICU care continued with the EVD in place through 10/01/12. The patient's neurological exams remained poor during the hospital stay. After a discussion with the family, the decision was made to provide comfort care only. The patient expired on 10/02/2012.

**CC-TBI-049** was a 23 year old male who was an unrestrained passenger in a motor vehicle accident on 10/20/12. The patient was reported as a GCS 3 on the scene. The patient was brought to Memorial Hermann and found to have diffused cerebral pneumocephalus, subdural hematoma, intraventricular hemorrhage and cistern obliteration. Neurosurgery placed a Camino Bolt at the bedside in the ICU (opening pressure of 25). On 10/22/2012 the patient was treated with hypothermia due to uncontrolled intracranial pressure. The patient's neurological exams remained poor during the hospital stay. After a lengthy discussion with the family, a decision was made to provide comfort care only. A DNR form was signed on 10/23/2012. The patient expired on 10/24/2012.



**CC-TBI-060** was a 58 year old female who was struck by a moving vehicle. The patient was a reported as a GCS 3 on the scene with blood pressure in the 60's. The patient was brought to Memorial Hermann and found to have shearing injuries to the left temporal lobe, right thalamus, and bilateral frontal lobes in addition to multiple trauma injuries. Neurosurgery placed a Camino Bolt at the bedside in STICU (opening pressure of 15). On 01/12/13 the patient's blood pressure dropped into the 70's. Cardiology diagnosed the patient with a STEMI. After a lengthy discussion with the family, a decision was made to transition to comfort care and discontinue all life support measures. A DNR form was signed on 01/12/13. The patient expired on 01/13/13 at 01:27.

**CC-TBI-068** was a 45 year old male who was involved in a motorcycle collision. The patient was brought to Memorial Hermann Hospital and found to have hemorrhagic shock as well as traumatic subarachnoid hemorrhage, large shear injury, and no brain stem reflexes. Neurosurgery placed an extra ventricular drain in the OR during an exploratory laparotomy (opening pressure of 68). A follow-up head CT showed increasing hemorrhages into the basal ganglia and brainstem. Due to the new developments, the neurosurgery team decided to discuss the poor prognosis with the family. Cerebral perfusion test was performed, which confirmed absent blood flow to the brain. Date and time of death was 3/28/13 at 23:03.

**CC-TBI-069** was a 64-year-old male who was admitted to Memorial Hermann Hospital on 03/30/13 after a motorcycle accident in which he was wearing a helmet, yet suffered a TSAH, SDH, cerebral edema, and extensive skull base fractures as demonstrated by the admit CT. After administration of mannitol an ICP monitor was placed on 03/30/13 at 20:15. Aggressive treatment of the patient's high ICP continued with no success. A DNR form was signed by the attending on 04/01/13. On 04/04/13, the patient's family decided to remove the ventilator. The patient expired from cardiac arrest on 04/04/13 at 12:50.

**CC-TBI-075** was a 61-year-old male involved in a motorcycle collision without a helmet on 4/19/2013. The patient was a GCS of 6 at the scene with an obvious head wound and a right open femur fracture. A subsequent head CT was performed on arrival showing bilateral subarachnoid hemorrhage, 1mm downward shift, and right parietal and right temporal bone fractures. An ICP monitor was placed at 09:30 on 4/20/13. The patient's condition and GCS score remained the same throughout the stay. On 4/29/13, the patient underwent surgery for femur fixation and wound vac change. After completion of surgery, he became bradycardic then asystolic. Full efforts were performed to bring heart rate back. After 18 minutes without return of electrical cardiac activity, the patient was pronounced dead at 14:08 on 4/29/13.

**CC-TBI-077** was a 39 year old male who was involved in an Auto/Pedestrian accident at highway speeds on 4/23/2013. The patient was brought to Memorial Hermann Hospital and found to be hypotensive and tachycardic. Neurosurgery placed an extra ventricular drain in the STICU (opening pressure of 22). A follow-up head CT demonstrated a worsening traumatic brain injury. The patient had negative gag, cough and corneal reflexes and his pupils were nonreactive. Due to the new developments, the neurosurgery team decided to discuss the poor prognosis with the family. Cerebral perfusion test was performed, which confirmed absent blood flow to the brain. Date and time of death was 4/30/13 at 15:00.

**CC-TBI-081** was a 46-year-old male with no reported prior past medical history who was initially admitted to the Memorial Hermann Hospital via Life Flight on 5/15/2013. The patient was reported to have been at the airport running on a treadmill when he was noted to fall and have a 5 to 10 minute CPR by a bystander. He was noted on arrival in the ER to be in very poor condition with bilateral fixed and dilated pupils. A head CT revealed a large right subdural hematoma with significant shift and the patient was taken emergently to the OR for decompression and clot evacuation. The patient's neurostatus failed to improve despite full management and the patient continued to have brain herniation and progressively

lost brainstem reflexes. After multiple meetings with the wife and mother, the decision was made to provide comfort care and he was extubated on 5/21/13. The patient was pronounced dead on 5/22/13.

**CC-TBI-083** is a 50-year-old male who was admitted to Memorial Hermann Hospital after a motorcycle accident on 5/19/13 in which the patient rear ended a truck. The patient was GCS14 on admission. Head CT revealed bilateral hemorrhagic contusions, left frontotemporal SDH, TSAH, and left frontal bone fractures. An EVD was placed and the patient was admitted to the NTICU. EVD was removed on 5/24/13 after monitor failure and the patient's improvement. Overnight on 5/25/13, the patient's neurological status declined (stupor). CT showed no changes. On 5/28/13, the patient was noted to be unresponsive to stimuli including negative brain stem reflexes. On 5/29/13, the patient went into PEA. He was coded with epinephrine and CPR was performed. The patient was pronounced brain dead at 14:13 on 5/29/13.

**CC-TBI-087** was a 38-year-old male who was admitted to Memorial Hermann Hospital after an auto-pedestrian accident on June 22, 2013 in which the patient was hit by two cars at high speeds. The patient was GCS of 3T at time of admission. Imaging was obtained to reveal that the patient had diffuse subarachnoid hemorrhage (SAH) and subdural hemorrhage (SDH). Neurosurgery was consulted who placed an extraventricular drain (EVD) in the Emergency Department. In the intensive care unit, the patient remained on a ventilator, off sedation with a persistently poor GCS. Repeated head CTs revealed no change to his head injuries. A family meeting was conducted and due to the patient's severe head injury and poor prognosis, the decision was made to provide comfort care only. He expired June 28, 2013 at 1720.

**CC-TBI-102** was a 59-year-old male who was admitted to Memorial Hermann Hospital after a motor-vehicle accident on 9/10/13. He was noted by EMS to have been partially ejected from the vehicle with his head through the driver window and against a tree. The patient was GCS 3T (T indicates intubation) upon admission. Imaging was obtained to reveal that the patient had right subarachnoid hemorrhage (SAH) and bilateral subdural hematoma (SDH) and hemorrhagic shearing of the corpus callosum. Neurosurgery was consulted and placed a Camino Bolt in the Shock Trauma Intensive Care Unit (STICU). Throughout the patient's hospital course, the patient was kept comfortable with appropriate pain and sedation medications. His blood pressures were treated aggressively to maintain his mean arterial pressure, adequate cerebral perfusion pressures, and measures were taken to keep his ICP below 20 mmHg. However, his cognitive function never recovered, and his GCS was never above a 5T. A meeting was held on 09/20/2013 with the family who decided to provide comfort measures only. The patient was pronounced dead on 9/20/2013.

**CC-TBI-106** was a 47-year-old female who was admitted to Memorial Hermann Hospital after auto-pedestrian accident on 9/14/13. The patient was a GCS of 3T upon admission. Imaging was obtained to reveal that the patient had SAH and intraventricular hemorrhage (IVH). Neurosurgery was consulted and placed a Camino Bolt in STICU. Throughout the patient's hospital course, the patient was kept comfortable with appropriate pain and sedation medications. The patient was treated prophylactically for seizure due to a subarachnoid hemorrhage. Her neurologic exam was remarkable for having a GCS of 4T with pathologic extensor posturing. The patient's intracranial pressures and cerebral perfusion pressures were monitored for the first few days and were all within normal limits. The physician spoke extensively with the patient's family about the poor prognosis, who decided to provide comfort measures only on 09/19/2013. The patient was pronounced dead on 9/20/2013.

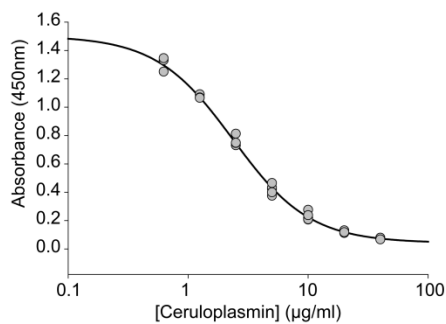
**CC-TBI-107** was a 23-year-old male who was admitted to Memorial Hermann Hospital after a motor vehicle accident on 9/20/13. The patient was a GCS of 3T upon admission. CT imaging revealed that the patient had acute SDH in both cerebral convexities, 10mm in the left and 9mm in the right. Initial ICP peaked at 98. NaCl 3% boluses reduced ICP to the 20-30. Craniectomy was eventually performed

with no positive results. Therapeutic hypothermia was used to control ICP as well. During rewarming phase, the patient developed respiratory distress and hypotension. Despite resuscitative efforts, the patient came to a cardiac standstill. Time of death was called on 09/27/13.

**CC-TBI-109** was a 20-year-old male with no significant past medical history who was admitted after a motor vehicle accident involving a rollover and ejection on 10/10/2013. The patient was found to have IVH, multifocal shear hemorrhages and diffuse axonal injury in bilateral frontal lobes, in the genu of the corpus callosum and in the right thalamus and pons, and a subarachnoid hemorrhage along the skull. He was managed with aggressive care in the shock trauma ICU after being admitted with an initial GCS of 4 in the field, requiring intubation via Life Flight. An ICP monitor was placed in the ICU. Overall, due to the poor prognostic factor from his extensive injuries, family members decided to withdraw aggressive care. Palliative care was administered to provide end-of-life symptom management and family support. The patient passed away on 10/16/2013.

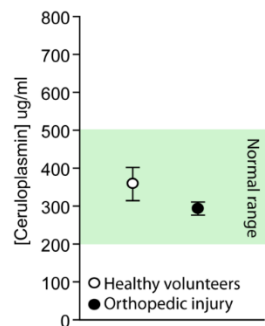
### **Assay characterization.**

The serum protein ceruloplasmin (also called iron(II):oxygen oxidoreductase) can be assayed using direct measures such as ELISAs and western blots, as well as indirect measures such as assessment of its iron oxidase activity. For these studies, our principal approach was to utilize an ELISA-based methodology based on its specificity, sensitivity, and ease of use. The assay used for the present study is a competitive ELISA in which a known amount of biotinylated ceruloplasmin competes for binding to an immobilized antibody with the ceruloplasmin in the sample. Figure 1 shows a representative standard curve for this assay demonstrating the inverse relationship between amount of unlabeled standard and the resultant optical density of the reaction product. Error across assays was found to be less than 10%, consistent with that reported by the vendor.



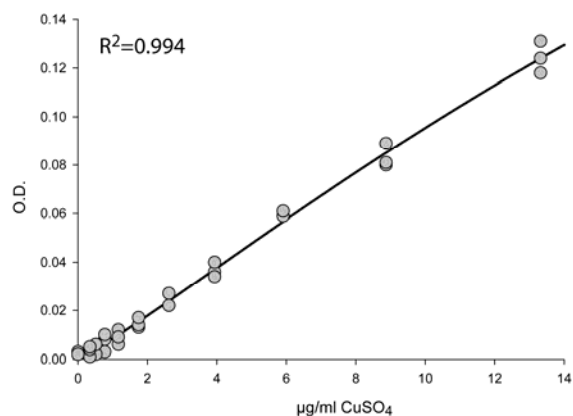
**Figure 1.** Relationship between amount of standard ceruloplasmin and optical density after ELISA.  $R^2$  of the curve was calculated to be 0.994.

Reference values for ceruloplasmin have been generally accepted to be 200-500 µg/ml (depending on the test), with levels below 200 µg/ml being indicative of pathology. Using plasma samples collected from healthy volunteers (n=13), we have found that the competitive ELISA employed returned a mean value of  $357.9 \pm 45.6$  µg/ml, well within the clinically accepted range (Figure 2). Representative healthy volunteer samples have been, and will continue to be, included on all subsequent assays to ensure assay performance and reproducibility. As an additional control, we have collected and initiated analysis of plasma samples collected from patients who have sustained an orthopedic injury, but have no self-reported or clinical manifestations of a head injury. As TBI often does not occur in isolation, inclusion of an orthopedic injury group will help us determine if the presence of other bodily injuries can give rise to clinically relevant decreases in ceruloplasmin levels. Figure 2 shows that although orthopedic injury patients (n=13) have modestly lower ceruloplasmin levels than that detected in healthy volunteers, these values remain in the normal range.



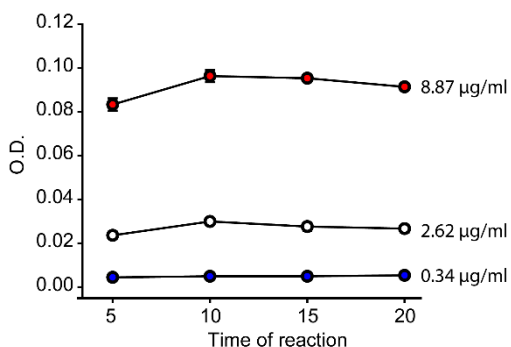
**Figure 2.** Ceruloplasmin levels in healthy volunteer and orthopedic injury controls. The serum ceruloplasmin levels for both the healthy volunteers (n=13) and orthopedic injury (n=13) groups was found to be within the normal range (green).

The copper assay to be employed uses 10.0  $\mu\text{g/ml}$  of 3,5-DiBr-PAESA in 0.2M acetate buffer (pH 5.0) containing 15mg/ml SDS and 35mM ascorbic acid. This solution is reacted with the sample for 5 min, after which the color product is quantified using a plate reader at 595nm. To assess the performance of this assay, sample analyses were carried out to determine the assay linearity, stability, and recovery. Figure 3 shows that in response to increasing concentrations of Copper(II) sulfate ( $\text{CuSO}_4$ ), the product generated by the reaction with 3,5-DiBr-PAESA follows a linear relationship. No absorbance of copper(II) sulfate at 595nm (when assessed in the absence of 3,5-DiBr-PAESA) was observed (data not shown).



**Figure 3.** Relationship between amount of copper(II) sulfate and optical density of product after reaction with 3,5-DiBr-PAESA. A linear relationship was observed between 0.3 $\mu\text{g/ml}$  and 13.3 $\mu\text{g/ml}$  ( $R^2=0.994$ ).

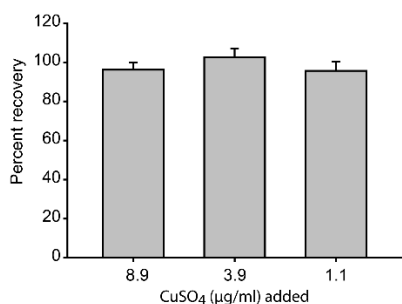
Satisfied by the linearity of the reaction, we next tested if the time of reaction contributes to the degree of product formed and/or its stability. Three concentrations of  $\text{CuSO}_4$  were tested, representing the high (8.87 $\mu\text{g/ml}$ ), middle (2.62 $\mu\text{g/ml}$ ), and low (0.34 $\mu\text{g/ml}$ ) ends of the linear range. Reaction times were increased in 5min intervals for up to 20min (4 times the normal incubation time). Figure 4 shows that the recorded optical density of the generated product remained constant over the entire range of incubation times.



**Figure 4.** Relationship between optical density and reaction time. There was no significant deviation in the optical density (O.D.) of the generated product across incubation times, indicating that the reaction is rapid, and that the generated product is stable.

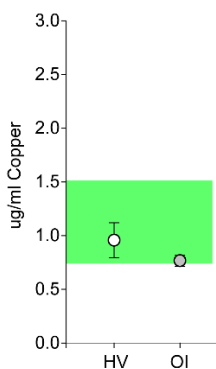
We next examined the percent recovery of exogenously added copper. Plasma from a healthy volunteer was spiked with different amounts of copper, then assayed. The calculated copper concentration from the

sample alone was subtracted from the resultant concentration, and the percent recovery calculated. Figure 5 shows that for all three copper concentrations utilized, recovery was greater than 95%, suggesting that the copper is freely available for reaction with 3,5-DiBr-PAESA. Taken together, these assay characterization results are consistent with our previous experience with this technique.



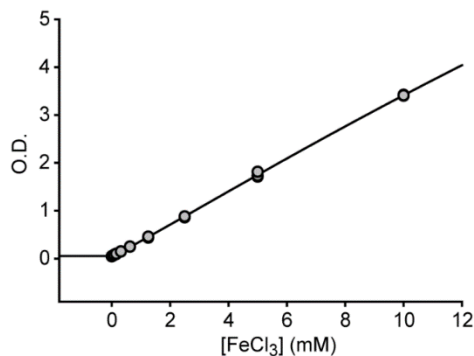
**Figure 5.** Recovery of exogenously added copper(II) sulfate to human plasma. Different amounts of CuSO<sub>4</sub> was added to a plasma sample then assayed for copper content. After subtracting the copper already present in the sample, percent recovery was calculated. Greater than 95% of all added copper was recovered.

Reference values for serum copper are 0.7-1.5 µg/ml. Using plasma samples collected from healthy volunteers (n=13), we have found that the copper assay employed gives a mean value of  $0.96 \pm 0.17$  µg/ml, well within the clinically accepted range (Figure 6). Similar to that seen for ceruloplasmin, when a group of orthopedic injury samples was analyzed, the levels of copper in these samples was found to be lower than those found in healthy volunteers, albeit still in the normal range (Figure 6).



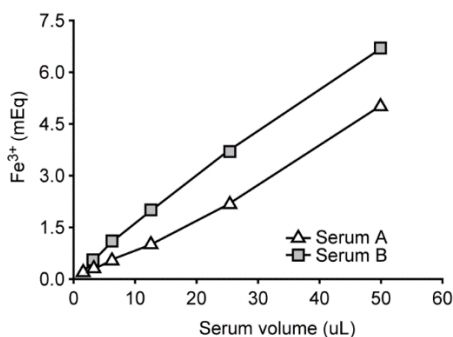
**Figure 6.** Copper levels in healthy volunteer and orthopedic injury controls. The serum copper levels for both the healthy volunteers and orthopedic injury groups was found to be within the normal range (green).

As described in the original proposal, ceruloplasmin activity can be an alternate methodology to measuring ceruloplasmin levels or copper. Ceruloplasmin is a copper-dependent iron oxidase which oxidizes iron from its ferrous ( $\text{Fe}^{2+}$ ) to ferric ( $\text{Fe}^{3+}$ ) state. Thus, the production of ferric iron from a ferrous iron substrate can be used as an indicator of ceruloplasmin activity/levels. To test the suitability of this assay as an alternative methodology, ceruloplasmin activity was assessed essentially as described by Boll et al., 1999. In brief, serum is incubated in a buffer containing 0.3M acetate buffer pH 6.0 and ammonium ferrous sulfate. The reaction is then deproteinated by the addition of 1.25M perchloric acid followed by centrifugation (10,000 X g for 3 min). The amount of generated ferric iron is determined by the addition of an equal volume of 0.5M ammonium thiocyanate and reading the optical density of the resultant solution at 450nm in a spectrophotometer. We first tested the linearity of this assay to detect increasing concentrations of ferric iron. Mock activity assays were carried out as described above with the exception that increasing amounts of  $\text{FeCl}_3$  was substituted for the serum. Figure 7 shows that the assay was linear to 10mM  $\text{FeCl}_3$ , yielding a standard curve with a  $R^2$  value of 0.999. This standard curve was subsequently used to determine the equivalents of ferric iron generated.



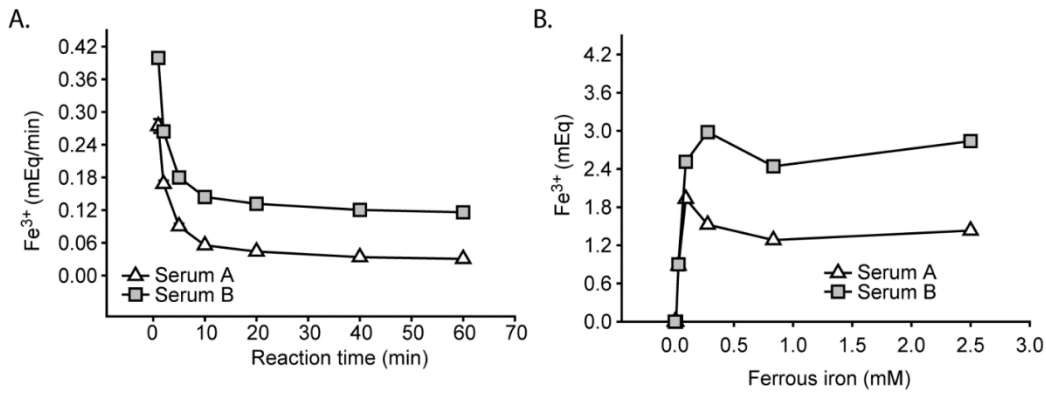
**Figure 7.** Standard curve showing the linear relationship between the amount of ferric iron added, and the optical density of the generated ferric thiocyanate product.

To determine the dose response relationship between the amount of ceruloplasmin and the generation of the ferric thiocyanate end-product, reactions were carried out in which the amount of starting material was varied from 0-50 $\mu$ l serum. Two different serum samples, having protein concentrations of 40mg/ml (serum A) and 65mg/ml (serum B), were tested. Figure 8 shows that there was a linear relationship between volume of serum and the amount of ferric iron produced between 5 and 50 $\mu$ l (Serum A,  $R^2 = 0.997$ ; Serum B,  $R^2 = 0.994$ ). Based on this result, a starting volume of 20 $\mu$ l serum was chosen for further studies.



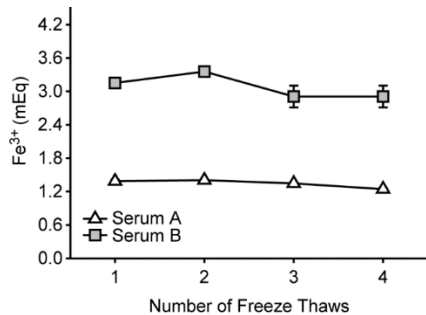
**Figure 8.** Relationship between the volume of serum added and the amount of ferric iron generated in the ceruloplasmin activity assay. Product formation was linear between 5 and 50 $\mu$ l starting material, with a calculated slope of 2.05.

We next examined the influence of reaction time on the rate of product generation. Assays were carried out in which the reaction time was varied from 1 to 60 min. The amount of ferric iron generated was calculated by comparison to a simultaneously prepared standard curve. The rate of product generation was calculated by dividing the amount of ferric iron generated by the time (in min) of the reaction. Figure 9A shows that the reaction rate was highest immediately upon addition of the substrate, dropping to a stable rate between 10 and 60 min. Based on this observation, a reaction time of 20 min was chosen. We also assessed the influence of substrate concentration on the amount of product generated to ensure that the substrate was sufficient to drive the reaction, but not in limiting amounts. Figure 9B shows that the amount of product generated was stable at substrate concentrations greater than 100 $\mu$ M. Based on this result, a substrate concentration of 1 mM was chosen for subsequent studies.



**Figure 9.** A) Rate of ferric iron production in relationship to reaction time. The reaction rate was stable between 10 and 60min in both samples assessed. B) Amount of product generated relative to starting material. The amount of product detected was consistent when the substrate concentration exceeded 100 $\mu$ M.

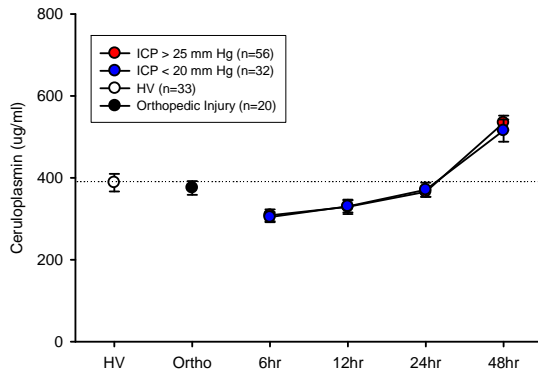
As the samples we propose to assay are processed then frozen at  $-80^{\circ}\text{C}$ , we questioned if the freeze thaw process would influence ceruloplasmin activity. To test this, serum samples were subjected to either 1, 2, 3 or 4 freeze-thaws prior to assay. Figure 10 shows that there was no demonstrable decrease in ceruloplasmin activity as a result of freeze thaw.



**Figure 10.** Influence of freeze-thaw of the serum sample on ceruloplasmin activity. Ceruloplasmin activity remained constant in samples subjected up to 4 freeze-thaw cycles.

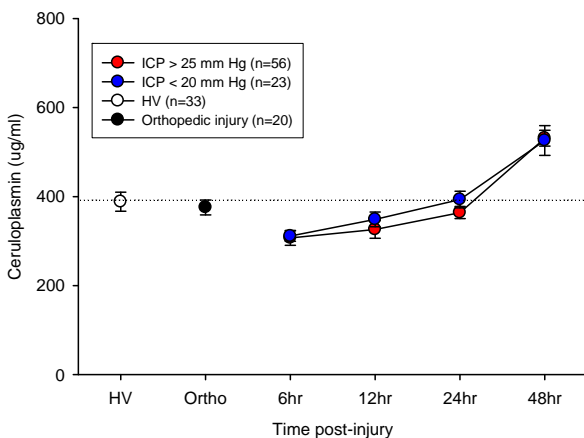
**Ceruloplasmin as a prognostic/diagnostic marker of elevated ICP.** In order to examine if ceruloplasmin has any prognostic/diagnostic value in identifying patients with elevated ICP, data collected over the first 48hr of injury was segregated based on patient classification and compared across groups. Groups of healthy volunteers (n= 33) and orthopedic injury patients (n=20) were assayed as non-brain injury controls.

i) All subjects. Figure 11 shows a summary of ceruloplasmin levels collected from all patients assayed (for clarity in presentation, and due to the small number of patients with ICP values between 20-25 mm Hg, the results from this group are not shown). As compared to healthy volunteers ( $H=27.003$ ,  $p \leq 0.001$ ) and orthopedic injury patients ( $H=27.492$ ,  $p \leq 0.001$ ), the level of ceruloplasmin is significantly lower in severe traumatic brain injury patients (independent of their ICP status) at 6hr and 12 hr after injury. This decrease occurred in both the elevated ICP (n=56) and non-elevated ICP (n=32) groups, and could not be used to differentiate between the patient populations (Figure 1). Similarly, both groups showed a delayed (> 24hr) increase in ceruloplasmin, a finding consistent with its role as an acute phase reactant protein.



**Figure 11. Ceruloplasmin levels by subject group.** Although an initial suppression, and a late-phase increase in ceruloplasmin were observed compared to healthy volunteers (HV) and orthopedic injury patients, there was no significant difference in ceruloplasmin levels between the TBI patients who had elevated ICP and those who did not.

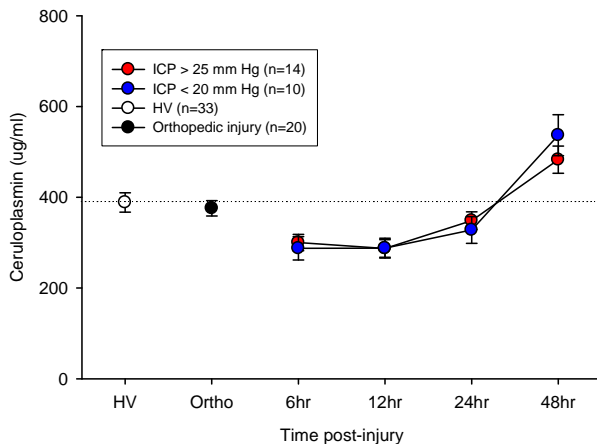
ii) Exclusion of ICP < 20mm Hg patients treated for ICP. As this was an observational study, patient care remained at the discretion of the attending physician. The ICP values assigned to each patient were recorded from an implanted ICP monitor used for standard patient care. As such, the ICP values recorded for each patient may have been influenced by ICP-controlling interventions such as craniectomy (removal of a bone flap to allow brain swelling), and administration of osmotic agents such as mannitol and/or hypertonic saline. We therefore excluded persons from the ICP <20 mm Hg group who had received one (or more) of these interventions. Figure 12 shows that the removal of patients whose low ICP values may have been the result of an intervention did not significantly alter the time course of ceruloplasmin, giving rise to mean values that were not different from those recorded in the ICP >25 mm Hg group.



**Figure 12. Exclusion of ICP ≤ 20 mm Hg patients who had ICP-controlling treatments does not improve the prognostic value of ceruloplasmin.** Patient records were audited to identify the subset of patients who had been treated with either mannitol, hypertonic saline, or had a craniectomy performed as a method to control ICP. When these patients were removed from the ICP ≤20 mm Hg group, no significant differences were observed between the TBI patients with elevated ICP and those without.

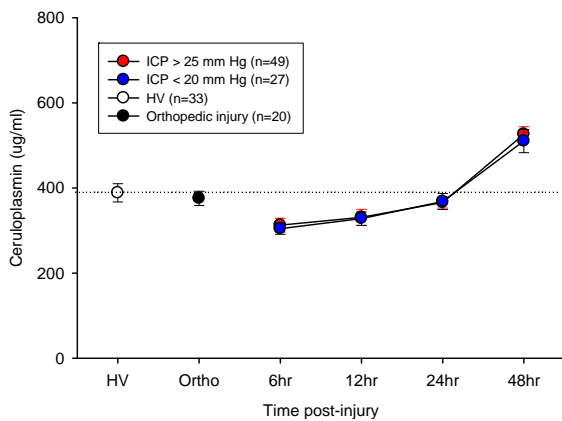
iii) Exclusion of patients with polytrauma. It has been reported that serum ceruloplasmin (as well as copper) levels are decreased (within 24hr of injury and persisting for days) in patients who have sustained thermal trauma (Shewmake et. al., 1988). Although none of our enrolled patients had significant thermal injuries, many did have injuries to other parts of the body. Since the levels of ceruloplasmin could be influenced by these peripheral injuries, we questioned if the prognostic/diagnostic value of this protein may have been compromised by the inclusion of polytrauma patients in our results. We therefore identified the TBI patients with isolated head trauma, and compared the recorded ceruloplasmin values between those with elevated ICP and those without. Figure 13 shows that when the patients with polytrauma were excluded from the analysis, ceruloplasmin levels still failed to segregate the patients based on their ICP status.





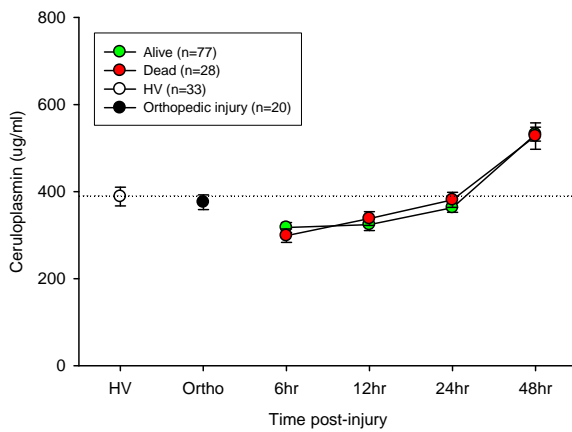
**Figure 13. In isolated head trauma, ceruloplasmin does not segregate low and high ICP patients.** The majority of severe TBI patients also present with extracranial injuries, a condition known as polytrauma. As these extracranial injuries may influence ceruloplasmin levels, patients with polytrauma were excluded from the analysis. Ceruloplasmin levels were not significantly different between the isolated head trauma patients who experienced elevated ICP and those whose ICP remained less than or equal to 20 mm Hg throughout the study period.

iv) Exclusion of patients who received blood products. Many TBI patients receive whole blood and/or fresh-frozen plasma (FFP) as a resuscitation fluid. If these products were given during the first 24 hr of injury, then they could have artificially “normalized” ceruloplasmin levels. To determine if the lack of differences seen between the ICP  $\geq 25$  mm Hg and ICP  $\leq 20$  mm Hg groups was due to the inclusion of subjects who had received blood/FFP transfusions, these patients were excluded from our analysis. Figure 14 shows that exclusion of the TBI patients who received blood products did not significantly alter the time course of ceruloplasmin suppression and subsequent elevation, nor did it reveal any significant differences between the ICP  $\geq 25$  mm Hg and ICP  $\leq 20$  mm Hg groups.



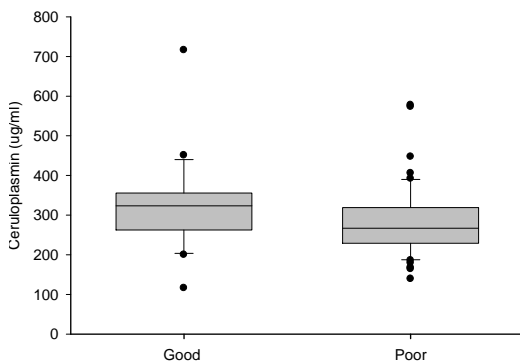
**Figure 14. Exclusion of patients resuscitated with plasma does not improve the prognostic value of ceruloplasmin.** As the ceruloplasmin present in donor plasma may mask post-TBI changes in ceruloplasmin, patients who received fresh-frozen plasma as a part of acute clinical care were removed from the analysis. In subjects that did not receive plasma, ceruloplasmin levels were not significantly different between TBI patients who experienced elevated ICP and those whose ICP remained less than 20 mm Hg throughout the study period.

v). Ceruloplasmin level as a predictor of mortality. Ceruloplasmin has been reported to be an independent predictor of all-cause mortality in patients with heart failure (Hammadah et al. 2014). Although we failed to corroborate our previous findings that ceruloplasmin may have prognostic value in identifying TBI patients at risk for developing elevated ICP, it had yet to be determined if the changes in the levels of this protein are indicative of outcome or survival. Figure 15 shows that when the TBI patients were segregated based on 30-day survival, there was no significant difference in ceruloplasmin levels between the patients who survived their injuries, and those who did not.



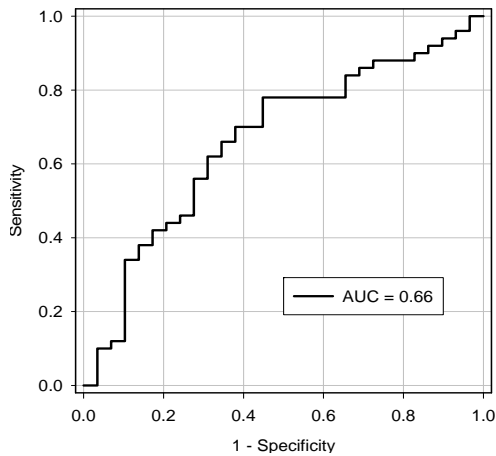
**Figure 15. Acute ceruloplasmin levels are not predictive of mortality after TBI.** Patients were stratified based on survival at the 30 day post-injury time point. Serum ceruloplasmin levels in survivors were not significantly different from those detected in non-survivors.

vi) Ceruloplasmin as a predictor of outcome. The Disability Rating Scale has been shown to be a reliable indicator of outcome after TBI, with testing performed at 1 month post-injury being highly correlated with outcome at 6 months (tested using the Glasgow Outcome Scale) (Clifton et al. 2011). Using a dichotomized scale of 0-6 (none-moderate disability) and 7-29 (moderately severe to vegetative), patients were separated based on their outcome. When the lowest recorded ceruloplasmin level (within the first 24hr of injury) was compared between patients with good outcome and those with poor outcome, ceruloplasmin was found to be modestly, but significantly, lower in patients with poor outcome ( $p=0.016$ ; Figure 16).



**Figure 16. Ceruloplasmin levels within 24hr of injury may be predictive of outcome.** Patients were grouped according to outcome as measured using the 30-day DRS score. Serum ceruloplasmin levels (lowest recorded within 24hr of injury) were found to be significantly reduced in subjects with poor outcome compared to those whose outcome was favorable. \*,  $p \leq 0.05$ .

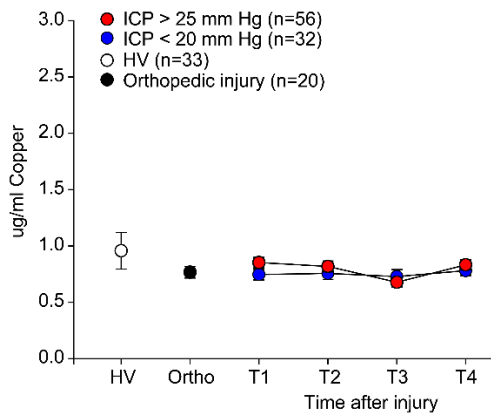
To determine if there is a cut-off value that could be used to predict which patients are likely to have a poor outcome, receiver operator characteristic (ROC) curve analysis was performed. As shown in Figure 17, the area under the curve (AUC) was calculated to be 0.66, making acute ceruloplasmin levels only a fair predictor of outcome. Using a cut-off value of 300 µg/ml, ceruloplasmin only had a sensitivity of 0.64 and a specificity of 0.66, suggesting limited clinical utility.



**Figure 17. ROC curve analysis indicates that acute post-injury ceruloplasmin levels have fair predictive value for outcome.** Sensitivity (true positive rate) and specificity (true negative rate) were calculated at multiple cut-off points. A plot of sensitivity versus 1-specificity gave rise to a curve with an AUC of 0.66. This suggests that ceruloplasmin has only limited clinical utility as an indicator of outcome.

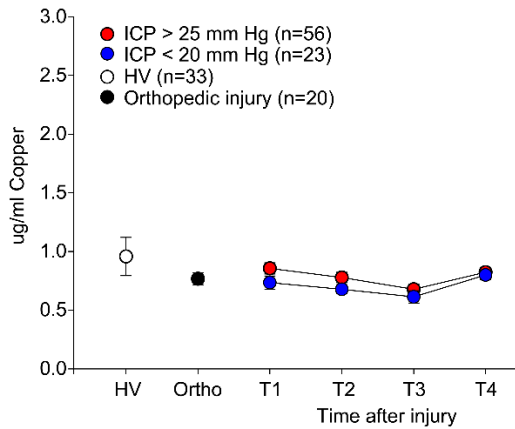
**Copper as a prognostic/diagnostic marker of elevated ICP.** As ceruloplasmin is the major copper binding protein in serum, copper levels may have prognostic capacity in predicting the occurrence of elevated ICP after TBI. Using a colorimetric assay, we assayed copper levels in the same samples collected for ceruloplasmin levels.

i). All subjects. Compared to the levels detected in healthy volunteers, serum copper was found to modestly decrease within the first 24hr of TBI. However, there was no significant difference when the TBI patients were stratified based on their ICP status (Figure 18). Interestingly, a decrease in serum copper was also observed in our orthopedic injury patients, suggesting that decreased copper levels may occur in response to bodily injuries.



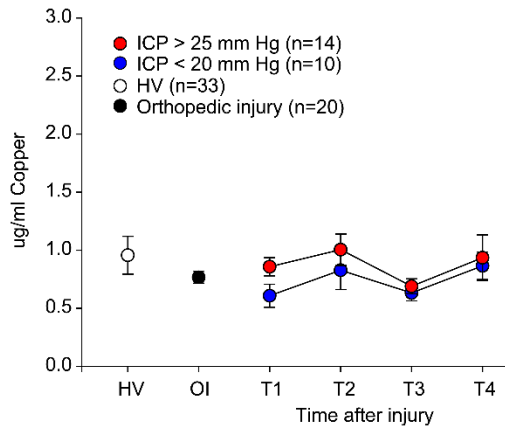
**Figure 18. Mean copper levels by subject group.** Although both the orthopedic injury and TBI patients had recorded copper levels that were modestly reduced compared to that seen in healthy volunteers, there was no significant difference in ceruloplasmin levels between the TBI patients who had elevated ICP and those who did not.

ii). Exclusion of ICP < 20mm Hg patients treated for ICP. To examine if ICP-controlling interventions may have led to ICP readings that could have resulted in the misclassification of subjects, we excluded subjects who underwent craniotomies to remove bone flaps, received mannitol, or were given hypertonic saline from the ICP  $\leq 20$ mm Hg group. Figure 19 shows that removal of patients whose low ICP values may have been the result of intervention did not significantly alter the time course of serum copper, giving rise to mean values that were not different from those recorded in the ICP  $\geq 25$ mm Hg group.



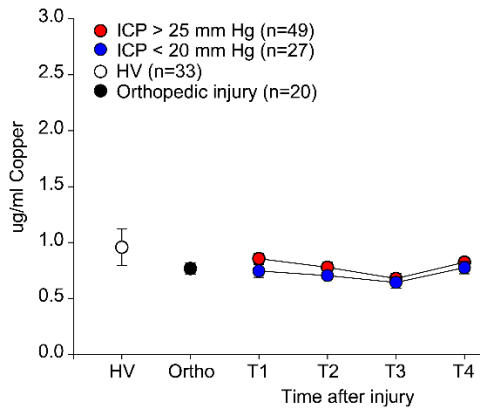
**Figure 19. Exclusion of ICP  $\leq$  20 mm Hg patients who had ICP-controlling treatments does not improve the prognostic value of copper.** Patient records were audited to identify the subset of patients who had been treated with either mannitol, hypertonic saline, or had a bone flap removed as a method to control ICP. When these patients were removed from the ICP  $\leq$  20 mm Hg group, no significant differences were observed between the TBI patients with elevated ICP and those without.

iii) Exclusion of patients with polytrauma. As mentioned above, both serum ceruloplasmin and copper have been reported to be decreased in response to peripheral injury, specifically thermal injury (Shewmake et. al., 1988). Further, our observation that orthopedic injury patients also have reduced serum copper levels suggests that its levels may be influenced by peripheral injuries. Figure 20 shows that when the patients with polytrauma were excluded from the analysis, copper levels still failed to differentiate patients based on their ICP status. Although copper levels at the T1 time point (i.e. 6hr post-injury) appear to be different between the two groups, exclusion of the polytrauma patients reduced the number of subjects (n=14 for ICP > 25 mm Hg and n=10 for ICP < 20 mm Hg) being interrogated (Fig 10). This may undermine the reproducibility of this finding.



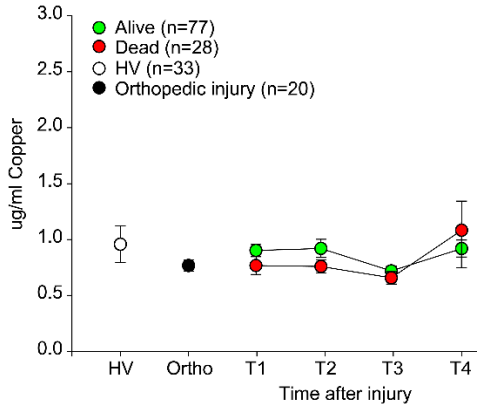
**Figure 20. Serum copper levels do not have prognostic value in isolated head trauma patients.** As extracranial injuries may influence serum copper levels, patients with polytrauma were excluded from the analysis. Serum copper levels were not significantly different between the isolated head trauma patients who experienced elevated ICP and those whose ICP remained less than 20 mm Hg throughout the study period.

iv) Exclusion of patients who received blood products. Based on the anticipation that copper levels would be normal in donated plasma, the administration of plasma/blood to our study subjects may have mitigated TBI-related changes. To assess this possibility, subjects who had received plasma/blood were excluded from our analysis. Figure 21 shows that exclusion of the TBI patients who received plasma did not reveal any significant differences between the ICP  $\geq$  25mm Hg and ICP  $\leq$  20mm Hg groups.



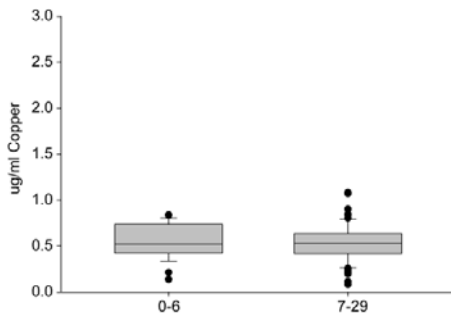
**Figure 21. Exclusion of patients resuscitated with plasma does not improve the prognostic value of copper.** As the copper present in donor plasma may confound measurements after TBI, patients who received plasma as a part of clinical care were removed from the analysis. In subjects that did not receive plasma, copper levels were not significantly different between TBI patients who experienced elevated ICP and those whose ICP remained less than or equal to 20 mm Hg throughout the study period.

v). Copper levels as a predictor of mortality. It has been suggested that post-TBI reductions in serum copper levels are associated with poorer outcome, possibly altering the activity of metalloenzymes such as Cu-Zn superoxide dismutase and metallothionin (Johary et. al., 2013). We therefore examined if serum copper levels in our patients could be used to predict 30-day mortality after TBI. Although there was a trend towards lower copper levels in patients who did not survive their injuries (Figure 22), this difference did not obtain statistical significance.



**Figure 22. Acute, post-injury copper levels are not predictive of mortality after TBI.** Patients were stratified based on survival at the 30 day post-injury time point. Serum copper levels were not significantly different in those who survived their injuries from those who did not.

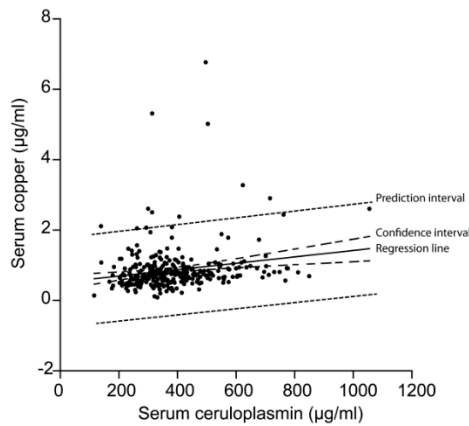
vi). Serum copper levels as a predictor of outcome. As indicated above, post-injury ceruloplasmin levels were found to be significantly lower in TBI patients with poor outcome (assessed using the Disability Rating Scale (DRS)) compared to values recorded in patients with none-to-moderate disabilities. To determine if copper levels could also be used to predict outcome, patients were separated into good (DRS = 0-6) and poor (DRS =7-29) outcomes. When the lowest recorded copper level within the first 24hr of injury was compared between the groups, no difference was detected (Figure 23).



**Figure 23. Copper levels within 24hr of injury are not predictive of outcome.** Patients were grouped according to outcome as measured using the 30-day DRS score. Serum copper levels (lowest recorded within 24hr of injury) were not found to be significantly different in subjects with poor outcome compared to those whose outcome was favorable. \*,  $p \leq 0.05$ .

## The combination of ceruloplasmin and copper as a prognostic/diagnostic biomarker signature for elevated ICP.

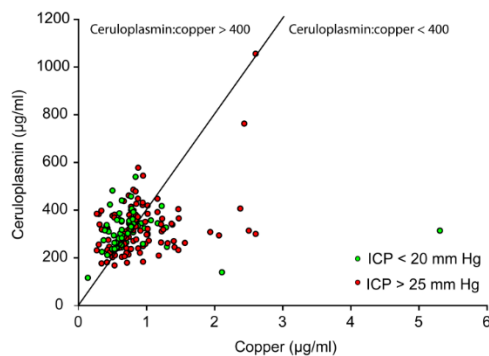
i) Linear regression. We first examined if there was a relationship between the recorded values for ceruloplasmin and copper levels. Linear regression indicated that there was a significant relationship between the two measures ( $F_{(1,319)} = 12.413$ ,  $p \leq 0.001$ ), although the curve fit ( $R^2$ ) was 0.038 (Figure 24). This indicates that although the measures trend together, they have limited predictive value for one another (as indicated by the large correlation confidence and prediction intervals). This suggest that, at least for some samples, ceruloplasmin and copper may be changing independent of one another.



**Figure 24. Scatter plot and linear regression for the serum levels of copper and ceruloplasmin.** Although both measures tended to change in the same direction, the levels of copper are not overtly predictive of ceruloplasmin levels.

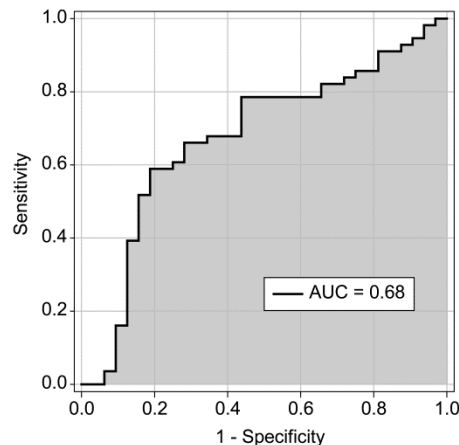
ii). Logistic regression. To determine if the combination of copper and ceruloplasmin has diagnostic value in stratifying TBI patients based on their ICP status, logistic regression was carried out. Samples were segregated based on their collection times into one of 4 groups: 0-12 hr (T1), 12-24 hr (T2), 24-36 hr (T3) and 36 – 48 hr (T4). In the T1 sample, logistic regression had a reasonable sensitivity, correctly identifying 41 of 48 samples as belonging to the  $ICP \geq 25$  mm Hg group. However, it had poor specificity, incorrectly identifying 19 of 28 samples from the  $ICP \leq 20$  mm Hg group as belonging to the high ICP group. There was no relationship between ICP status and the levels of ceruloplasmin and copper for any of the other time intervals.

iii). Ceruloplasmin-to-copper ratio. Although logistic regression did not reveal any meaningful relationship between ICP status and the combination of copper and ceruloplasmin, scatter plot analysis of the recorded values within the first 24 hr suggested that the ratio of ceruloplasmin:copper may be reduced in samples from patients whose  $ICP \geq 25$  mm Hg versus those whose  $ICP \leq 20$  mm Hg (Figure 25). In order to assist in visualizing the distribution, a line was drawn to indicate a 400:1 ceruloplasmin:copper ratio. It appears that more patients with a low ceruloplasmin:copper ratio ( $\leq 400$ ) belong to the  $ICP \geq 25$  mm Hg group.



**Figure 25. Scatter plot of ceruloplasmin versus copper values within first 24 hr of injury.** Data was segregated based on ICP status. A line was drawn to indicate a 400:1 ceruloplasmin:copper ratio.

We therefore calculated the ceruloplasmin:copper ratio for each sample, and then used these ratios to carry out Receiver Operator Characteristic (ROC) curve analysis. The lowest ratio recorded during the first 24 hr of injury was used. The ratio of ceruloplasmin:copper was found to only have an area under the curve (AUC) of 0.68 (Figure 26), making it only a poor predictor of ICP status. Using a cut-off of 400, the ceruloplasmin:copper ratio had a sensitivity of 79%, but a specificity of only 56%. Although there appears to be a weak relationship between ICP status and the ratio of ceruloplasmin:copper, the differences in the ratios between the ICP  $\leq$  20mm Hg and the ICP  $\geq$  25mm Hg group are too small to be of any practical utility.



**Figure 26. ROC curve showing the prognostic ability of the ceruloplasmin:copper ratio to identify patients with elevated ICP.** The calculated area under the curve (AUC) was found to be 0.68, making the ceruloplasmin:copper ratio a poor indicated of ICP status.

### 3c. What opportunities for training and professional development has the project provided?

Nothing to Report

### 3d. How were the results disseminated to communities of interest?

The results from the current project demonstrated that although severe TBI causes an acute decrease in both copper and ceruloplasmin levels, followed by a delayed increase, these levels did not significantly differ between patients who developed elevated ICP and those who did not. Due to the negative nature of these findings, the results from this study have not been published or disseminated to the public.

### 3e. What do you plan to do during the next reporting period to accomplish the goals?

The project is completed.

## 4. IMPACT:

### 4a. What was the impact on the development of the principal discipline(s) of the project?

Although plasma ceruloplasmin and copper were found to have no significant diagnostic/prognostic utility for TBI-associated elevated intracranial pressure, our findings that ceruloplasmin levels decrease acutely after severe TBI may have therapeutic implications. One of the common consequences of TBI is the increased production of free radicals. Free radicals such as the hydroxyl radical are generated by the chemical reaction of hydrogen peroxide (produced as a result of cellular respiration) and ferrous ( $\text{Fe}^{2+}$ ) iron. Free radicals are highly reactive and non-specifically oxidize proteins and other cellular components, causing cell damage and even death. Ceruloplasmin is a major iron(II):oxygen oxidoreductase that oxidizes iron from the ferrous ( $\text{Fe}^{2+}$ ) to the ferric ( $\text{Fe}^{3+}$ ) state. Thus, a reduction in ceruloplasmin levels may contribute to free radical production by allowing more iron to stay in the reactive  $\text{Fe}^{2+}$  state.

### 4b. What was the impact on other disciplines?

Nothing to Report.

#### **4c. What was the impact on technology transfer?**

Nothing to Report.

#### **4d. What was the impact on society beyond science and technology?**

Nothing to Report

### **5. CHANGES/PROBLEMS:**

#### **5a. Changes in approach and reasons for change**

There were no changes in the approved approach during this project.

#### **5b. Actual or anticipated problems or delays and actions or plans to resolve them**

During the course of the project, there were unanticipated delays in subject recruitment. Our initial estimates for enrolling subjects was based on the number of severe TBI patients seen at Memorial Hermann Hospital in the Texas Medical center (physically connected to the University of Texas medical School) and historic data on the rate of ICP monitor implantations. However, a change in practice at Memorial Hermann Hospital resulted in a reduction in the number of patients being implanted with ICP monitors, slowing our recruitment efforts. As a result, no-cost extensions were required to complete our patient enrollment and carry out our proposed ceruloplasmin and copper measurements.

#### **5c. Changes that had a significant impact on expenditures**

The delay in recruiting study subjects caused an unanticipated reduction in expenditures, as funds to be used for carrying out biomarker analysis were not expended as projected. No-cost extensions were requested, and granted, allowing the funds to be utilized as intended once patient enrollment was completed.

#### **5d. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

During the course of this study, two change requests were approved by our Institutional Review Board and the Human Research Protection Office (HRPO). These changes were not implemented until approval was granted.

In the first request, permission was sought to omit the collection of early samples, as long as a 24hr post-injury sample could be collected. The study was approved for blood samples at 4 time points (as close to injury as possible, 12hrs post-; 24hrs post- and 2-5days post-injury). If a subject could be enrolled and sample drawn within 24 hours (possibly omitting the first time points), we requested permission to include this subject. Allowing the inclusion of subjects where the early samples were not available (e.g., the decision to place an ICP monitor was made late) provided us valuable samples to support the aims of the study and aided in boosting enrollment. Also included in this request was to add a time point at greater than or equal to 14 days post-injury if the patient was available. We did not request that the patient return specifically for this visit, rather we attempted to collect the sample at a routine follow-up visit or if the patient was still an inpatient.

In the second change request, we sought permission to utilize two sets of patient samples from study subjects that could be provide consent. The approved protocol permitted collection of blood samples at 4 time points, the first within approximately 6 hours of injury, and allowed consent to be obtained later. The study attempted to obtain consent as soon as possible so as to honor patient/family wishes and not be involved or expend resources in the event of participation being declined. Samples from these subjects were processed and are stored, labeled with a study number and study time point. No identifiers were included on the samples. The two subjects without consent suffered severe traumatic brain injury and were unable to provide informed consent. Legally authorized representatives were not available to provide consent. The first patient with un-



consentable mental status and no LAR present was transferred to another hospital in Mexico within the first two weeks of injury. We worked with the hospital liaison to obtain consent from the LAR, but the LAR was not able to obtain permission to enter the US prior to the patient's transfer to Mexico and therefore we were unable to conduct the consent process. The second patient (mental status remained unconsentable greater than 30 days post injury) had no family available to provide consent. Repeated attempts were made to meet with a cousin at Memorial Hermann and TIRR hospitals 10/7/11; 10/10/11; 10/12/11; 10/17/11. On three occasions, the patient's cousin agreed to come to the hospital in the evening and then did not show up at the pre-arranged time. Study personnel continued to work with hospital personnel at both MHH and TIRR who indicated no one was visiting the patient. Because of the unusual circumstances of these cases, we were granted permission to keep the blood samples obtained in good faith and to assay them for our approved research question.

No vertebrate animals, biohazards, or select agents were used as a part of this study.

## 6. PRODUCTS:

### 6a. Publications, conference papers, and presentations

Nothing to Report

### 6b. Website(s) or other Internet site(s)

Nothing to Report

### 6c. Technologies or techniques

Nothing to Report

### 6d. Inventions, patent applications, and/or licenses

Nothing to Report

### 6e. Other Products

As a result of this project, plasma samples and medical data were collected and archived from a total of 110 persons who had sustained a severe TBI. These samples were used in conjunction with previously archived samples collected from age, gender, and race-matched healthy volunteers, and patients who had sustained an orthopedic injury to determine the consequences of severe TBI on plasma ceruloplasmin and copper levels.

## 7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

**7a. What individuals have worked on the project?** The following is a cumulative effort for all personnel from 12/1/2010 to 11/30/2015.

Name:	Pramod Dash
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	6 months
Contribution to Project:	Dr. Dash conceived and designed the study. He was responsible for determining the allocation of resources for this project, for trouble-shooting any technical

	problems, and for interpreting study results. Dr. Dash directly contributed to the copper and ceruloplasmin measurements.
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Name:	Georgene Hergenroeder
Project Role:	Collaborator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	10 months
Contribution to Project:	Ms. Hergenroeder served as the liason between the medical care staff and laboratory personnel. She was responsible for obtaining all regulatory approvals to initiate the project including preparing all the necessary IRB and HRPO documentation. Ms. Hergenroeder was also responsible for maintaining all the patient data, for ensuring accurate data entry, for informing the medical staff of Memorial Hermann Hospital about the research study, and for supervising the personnel obtaining consent.

Name:	Imoigele Aisiku
Project Role:	Collaborator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.0 month
Contribution to Project:	As a neuro-intensivist, Dr. Aisiku provided clinical care for many of the severe TBI patients used in this study. He served as the safety monitor for the study.

Name:	Jose Barrerra
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	43 months
Contribution to Project:	Mr. Barrerra was responsible for obtaining and processing the collected blood samples, for consenting the patient (or their next of kin), and for entering relevant medical data into the database.

Name:	Travis Shields
Project Role:	Research Assistant
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	21 months
Contribution to Project:	Mr. Shields was responsible for obtaining and processing the collected blood samples, for consenting the patient (or their next of kin), and for entering relevant medical data into the database.

Name:	Cameron Jeter
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	13 months
Contribution to Project:	Dr. Jeter was responsible for obtaining and processing the collected blood samples, for consenting the patient (or their next of kin), and for entering relevant medical data into the database.

Name:	Long Huang
Project Role:	Program analyst II
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	5 months
Contribution to Project:	Mr. Huang was responsible for establishing the databases for collecting the medical data used in this study. Further, he was responsible for conducting audits of the database to ensure the fidelity of the transferred data.

Name:	Sara Orsi
Project Role:	Sr. Research Associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	16 months

Contribution to Project:	As laboratory manager for Dr. Dash (2011-2012), Ms. Orsi was responsible for ordering all the supplies required for the completion of the research project. This included, but is not limited to, blood collection tubes, ELISA kits, chemicals, gloves, and plasticware.
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Name:	Kimberly Hood
Project Role:	Research Associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	27 months
Contribution to Project:	As laboratory manager for Dr. Dash (2012-2015), Ms. Hood was responsible for ordering all the supplies required for the completion of the research project. This included, but is not limited to, blood collection tubes, ELISA kits, chemicals, gloves, and plasticware.

Name:	John Redell
Project Role:	Research Scientist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	15 months
Contribution to Project:	Dr. Redell was responsible for carrying out the ceruloplasmin ELISA and copper assays.

Name:	Anthony Moore
Project Role:	Research Coordinator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	41 months
Contribution to Project:	Mr. Moore was responsible for carrying out the ceruloplasmin ELISA and copper assays.

**7b. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to Report

**7c. What other organizations were involved as partners?**

*Organization Name:* Memorial Hermann Hospital

*Location of Organization:* Houston, Texas, USA

*Partner's contribution to the project:* Patient consent and recruitment was carried out in Memorial Hermann Hospital. Sample collection (i.e. blood withdrawal) was carried out in Memorial Herman Hospital. Medical data recorded by attending physicians and nurses was collected by study personnel for use in this project.

**8. SPECIAL REPORTING REQUIREMENTS**

Nothing to Report

**9. APPENDICES:**

Not included.