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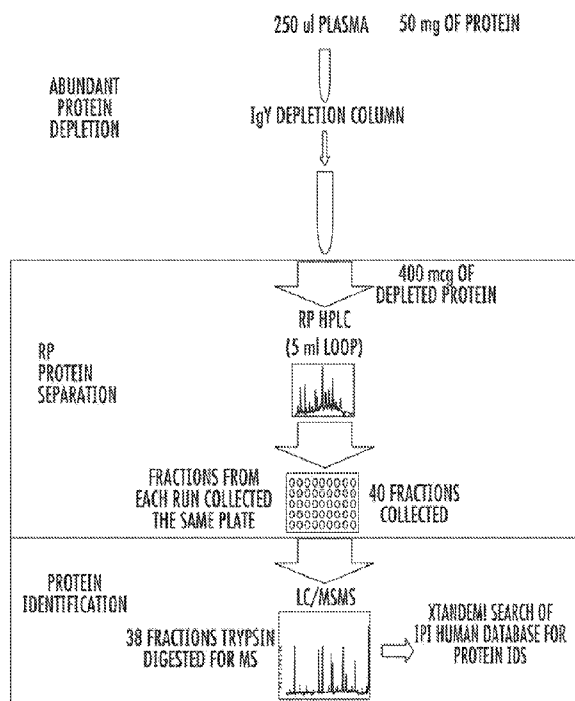
(54) Title: BIOMARKERS OF BRAIN INJURY

FIG. 1

(57) Abstract: The present invention relates to the field of biomarkers. More specifically, the present invention relates to biomarkers useful in diagnosing brain injuries. Brain injury can include overt or traumatic brain injury, as well as subclinical brain injury (SCI). In one embodiment, a method for diagnosing SCI in a patient comprises (a) collecting a sample from the patient; (b) measuring the levels of a panel of biomarkers in the sample collected from the patient, wherein the panel of biomarkers comprises ASTN1, BAI3, CNDP1, ERMIN, GFAP, GRM3, KLH32, MAGE2, NRG3, NRGN, OMG, SLC39A12, RTN1, and MT3; and (c) comparing the levels of the panel of biomarkers with predefined levels of the same panel of biomarkers that correlate to a patient having SCI and predefined levels of the same panel of biomarkers that correlate to a patient not having SCI, wherein a correlation to one of the predefined levels provides the diagnosis.

UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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BIOMARKERS OF BRAIN INJURY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 61/436,956,

5   filed January 27, 2011; U.S. Provisional Application No. 61/436,955, filed January, 27, 2011,

and U.S. Provisional Application No. 61/393,009, filed October 14, 2010, all of which are

incorporated herein by reference in their entireties.

STATEMENT OF GOVERNMENTAL INTEREST

This invention was made with U.S. government support under grant no. NHLBI 1

10   R01 HL091759-02, and NHLBI 5U54HL090515-02. The U.S. government has certain rights

in the invention.

FIELD OF THE INVENTION

The present invention relates to the field of biomarkers. More specifically, the

present invention relates to biomarkers useful in diagnosing brain injuries.

15   ### INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED

ELECTRONICALLY

This application contains a sequence listing. It has been submitted electronically via

EFS-Web as an ASCII text file entitled "P10784-08_Sequence_Listing-ST25.txt." The

sequence listing is 1,947 bytes in size, and was created on October 13, 2011. It is hereby

20   incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

Brain injuries are complex and can have multiple severe clinical outcomes. Injury of

the brain and spinal cord can result from head trauma, stroke, traumatic birth, heart surgery,

cardiac arrest and patients requiring cardiovascular support with ventricular assist devices or

25   extracorporeal membrane oxygenation (ECMO). Moreover, detection of subclinical brain

injury is difficult, especially in children and neonates with birth-related injury. Children with

sickle cell disease are at high risk for subclinical brain injury. Untreated subclinical brain

injuries in children can progress to overt stroke, neurological damage, learning problems and

memory loss.

30   Unfortunately, clinical tools such as physical exam, and imaging (CT Scan or MRI)

are subjective, not widely available, not sensitive or specific enough and or too costly to

identify the infant, child or adult with brain injury. There is a great clinical need to identify

patients with brain injury and especially subclinical injury because these infants, children and

adults are at significant risk of progressing to overt stroke and development of cognitive and motor loss, and dementia.

SUMMARY OF THE INVENTION

The present invention is based, at least in part, on the discovery of central nervous system (CNS)-specific protein biomarkers circulating in body fluids after brain and CNS injury. Release or secretion of proteins from cells of the CNS can be useful for diagnostic/prognostic assessment of patient viability, recovery and the effects of therapy to stabilize or prevent new or recurrent CNS injury in children and adults. Thus, detection of circulating CNS proteins improves the diagnostic accuracy of CNS injury by identifying children and adults with subclinical and overt brain injury and provides the means to determine and validate new and existing CNS injury treatments for efficacy to improve outcomes. This would also provide a means for screening new protocols or drugs (interventions) for reduced injury.

Accordingly, in one aspect, the present invention provides biomarkers useful for diagnosing children and adults with brain injury. In one embodiment, the brain injury is subclinical brain injury (SCI). In another embodiment, brain injury is overt brain injury. The biomarkers can be used to diagnose patients, assess a patient's prognosis over time, or generally determine a patient's brain injury status.

In particular embodiments, a method for diagnosing brain injury in a patient comprises the steps of (a) collecting a sample from the patient; (b) measuring the levels of one or more biomarkers in the sample collected from the patient; and (c) comparing the levels of the one or more biomarkers with predefined levels of the same biomarkers that correlate to a patient having brain injury and predefined levels of the same biomarkers that correlate to a patient not having brain injury, wherein a correlation to one of the predefined levels provides the diagnosis.

In more specific embodiments, a method for diagnosing subclinical brain injury (SCI) in a patient comprises the steps of (a) collecting a sample from the patient; (b) measuring the levels of one or more biomarkers in the sample collected from the patient; and (c) comparing the levels of the one or more biomarkers with predefined levels of the same biomarkers that correlate to a patient having SCI and predefined levels of the same biomarkers that correlate to a patient not having SCI, wherein a correlation to one of the predefined levels provides the diagnosis.

The one or more biomarkers can be selected from the group consisting of astrotactin 1 (ASTN1), brain angiogenesis inhibitor 3 (BAI3); carnosine dipeptidase 1 (CNDP1); ERMIN;

glial fibrillary acidic protein (GFAP); glutamate receptor metabotropic 3 (GRM3); kelch-like protein 32 (KLH32); melanoma antigen family E,2 (MAGE2); neuregulin 3 (NRG3); neurogranin (NRGN); oligodendrocyte myelin glycoprotein (OMG); solute carrier family 39 (zinc transporter), member 12 (SLC39A12); reticulon 1 (RTN1); and metallothionein (MT3).

5   In a specific embodiment, the one or more biomarkers comprises NRGN. In another embodiment, the one or more biomarkers comprises OMG. In yet another embodiment, the one or more biomarkers comprises MT3. In a further embodiment, the one or more biomarkers further comprises GFAP.

In another embodiment, the one or more biomarkers comprise NRGN, OMG, and
10   MT3. The biomarkers can further comprise GFAP. In yet another embodiment, the one or more biomarkers further comprise the biomarkers listed in Table 1 and Table 2.

In another embodiment, a method for diagnosing brain injury in a patient comprises the steps of (a) collecting a sample from the patient; (b) measuring the levels of a panel of biomarkers in the sample collected from the patient, wherein the panel of biomarkers
15   comprises ASTN1, BAI3, CNDP1, ERMIN, GFAP, GRM3, KLH32, MAGE2, NRG3, NRGN, OMG, SLC39A12, RTN1, and MT3; and (c) comparing the levels of the panel of biomarkers with predefined levels of the same panel of biomarkers that correlate to a patient having brain injury and predefined levels of the same panel of biomarkers that correlate to a patient not having brain injury, wherein a correlation to one of the predefined levels provides
20   the diagnosis.

In yet another embodiment, a method for diagnosing SCI in a patient comprises the steps of (a) collecting a sample from the patient; (b) measuring the levels of a panel of biomarkers in the sample collected from the patient, wherein the panel of biomarkers comprises ASTN1, BAI3, CNDP1, ERMIN, GFAP, GRM3, KLH32, MAGE2, NRG3,
25   NRGN, OMG, SLC39A12, RTN1, and MT3; and (c) comparing the levels of the panel of biomarkers with predefined levels of the same panel of biomarkers that correlate to a patient having SCI and predefined levels of the same panel of biomarkers that correlate to a patient not having SCI, wherein a correlation to one of the predefined levels provides the diagnosis.

In an alternative embodiment, a method for diagnosing brain injury in a patient
30   comprises the steps of (a) collecting a sample from the patient; (b) measuring the levels of a panel of biomarkers in the sample collected from the patient, wherein the panel of biomarkers comprises NRGN and GFAP; and (c) comparing the levels of the panel of biomarkers with predefined levels of the same biomarkers that correlate to a patient having brain injury and predefined levels of the same biomarkers that correlate to a patient not having brain injury,

wherein a correlation to one of the predefined levels provides the diagnosis. In a more specific embodiment, the panel of biomarkers further comprises OMG, and MT3. In a further embodiment, the panel of biomarkers further comprises ASTN1, BAI3, CNDP1, ERMIN, GRM3, KLH32, MAGE2, NRG3, OMG, SLC39A12, RTN1, and MT3.

5   In a further embodiment, a method for diagnosing SCI in a patient comprises the steps of (a) collecting a sample from the patient; (b) measuring the levels of a panel of biomarkers in the sample collected from the patient, wherein the panel of biomarkers comprises NRGN and GFAP; and (c) comparing the levels of the panel of biomarkers with predefined levels of the same biomarkers that correlate to a patient having SCI and predefined levels of the same

10   biomarkers that correlate to a patient not having SCI, wherein a correlation to one of the predefined levels provides the diagnosis. In a more specific embodiment, the panel of biomarkers further comprises OMG, and MT3. In a further embodiment, the panel of biomarkers further comprises ASTN1, BAI3, CNDP1, ERMIN, GRM3, KLH32, MAGE2, NRG3, OMG, SLC39A12, RTN1, and MT3.

15   In another embodiment, a method for diagnosing brain injury in a patient comprises the steps of (a) collecting a plasma sample from the patient; (b) measuring the levels of a panel of biomarkers in the plasma sample collected from the patient, wherein the panel of biomarkers comprises NRGN and GFAP; and (c) comparing the levels of the panel of biomarkers with predefined levels of the same biomarkers that correlate to a patient having

20   brain injury and predefined levels of the same biomarkers that correlate to a patient not having brain injury, wherein a correlation to one of the predefined levels provides the diagnosis. In a more specific embodiment, the panel of biomarkers further comprises OMG, and MT3. Alternatively, the panel of biomarkers further comprises ASTN1, BAI3, CNDP1, ERMIN, GRM3, KLH32, MAGE2, NRG3, OMG, SLC39A12, RTN1, and MT3.

25   In yet another embodiment, a method for diagnosing SCI in a patient comprises the steps of (a) collecting a plasma sample from the patient; (b) measuring the levels of a panel of biomarkers in the plasma sample collected from the patient, wherein the panel of biomarkers comprises NRGN and GFAP; and (c) comparing the levels of the panel of biomarkers with predefined levels of the same biomarkers that correlate to a patient having SCI and

30   predefined levels of the same biomarkers that correlate to a patient not having SCI, wherein a correlation to one of the predefined levels provides the diagnosis. In a more specific embodiment, the panel of biomarkers further comprises OMG, and MT3. Alternatively, the panel of biomarkers further comprises ASTN1, BAI3, CNDP1, ERMIN, GRM3, KLH32, MAGE2, NRG3, OMG, SLC39A12, RTN1, and MT3.

In another embodiment of the present invention, a method for determining the brain injury status in a patient comprises the steps of (a) collecting a sample from the patient; (b) measuring the levels of a panel of biomarkers in the sample collected from the patient, wherein the panel of biomarkers comprises ASTN1, BAI3, CNDP1, ERMIN, GFAP, GRM3, KLH32, MAGE2, NRG3, NRGN, OMG, SLC39A12, RTN1, and MT3; and (c) comparing the levels of the panel of biomarkers with predefined levels of the same panel of biomarkers that correlate to one or more brain injury statuses selected from the group consisting of having brain injury, not having brain injury, progressing brain injury, and regressing brain injury, wherein a correlation to one of the predefined levels determines the brain injury status of the patient.

In particular embodiments of the present invention, a method for determining the SCI status in a patient comprises the steps of (a) collecting a sample from the patient; (b) measuring the levels of a panel of biomarkers in the sample collected from the patient, wherein the panel of biomarkers comprises ASTN1, BAI3, CNDP1, ERMIN, GFAP, GRM3, KLH32, MAGE2, NRG3, NRGN, OMG, SLC39A12, RTN1, and MT3; and (c) comparing the levels of the panel of biomarkers with predefined levels of the same panel of biomarkers that correlate to one or more SCI statuses selected from the group consisting of having SCI, not having SCI, progressing SCI, and regressing SCI, wherein a correlation to one of the predefined levels determines the SCI status of the patient.

In particular embodiments of the present invention, the measuring step comprises immunoassay, immunoblotting method, immunoprecipitation assay, immunostaining method, quantitative assay, immunofluorescent assay, or a chemiluminescence assay. In certain embodiments, the sample is a blood, plasma serum, cerebrospinal fluid (CSF), or urine sample. In a specific embodiment, the sample is a blood sample. In a more specific embodiment, the sample is a serum sample.

In another aspect, the present invention provides kits for determining brain injury status in a patient. In particular embodiments, the diagnostic kit comprises a substrate for collecting a biological sample from the patient; and means for measuring the levels of one or more biomarkers selected from the group consisting of ASTN1, BAI3, CNDP1, ERMIN, GFAP, GRM3, KLH32, MAGE2, NRG3, NRGN, OMG, SLC39A12, RTN1, and MT3.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is an overview of the approach for plasma depletion, RP-HPLC separation and MS analysis.

FIG. 2 shows the spectra ion table for GFAP peptide. The pepP probability was 0.7598 and the XCORR 4.016. This peptide was found in 4 of 6 SCI patients.

FIG. 3 displays Plasma GFAP concentrations in steady-state sickle cell disease (n=259, HbSS and HbSβ^0, 5-14 years old), healthy children (n=47), and non-sickle cell in patients who had acute stroke (20 samples from 12 patients), brain biopsy (6 samples from 3 patients), or partial brain resection (22 samples from 13 patients). The dashed line marks the 95th percentile value among 47 healthy controls age-matched to the SITT cohort.

FIG. 4 displays plasma concentrations of GFAP in steady-state sickle cell disease, according to SCI status (n=66 SCI positive, n=145 SCI negative). The dashed line marks the 95th percentile value among 47 healthy, age-matched controls.

FIG. 5 shows serial plasma GFAP levels in an 11 year-old patient with HbSS and acute stroke. A red cell exchange transfusion was performed 5 hours after clinical diagnosis, and again 47 hours later for new neurologic deficits. The patient's plasma GFAP remained elevated at 0.50 ng/ml 11 days after diagnosis and returned to a normal value of 0.074 ng/ml 26 days later, immediately prior to the first scheduled red cell transfusion. The dashed line marks the 95th percentile value among 47 healthy, age-matched controls.

FIG. 6 displays neuroimaging of acute stroke in an HbSS patient who had plasma GFAP followed serially. Left: Non-contrast head CT immediately after clinical diagnosis of stroke showing a wedge of hypoattenuation in the right frontal lobe. Right: FLAIR MRI image 29 hours after diagnosis of stroke showing evolving right anterior cerebral artery infarct.

FIG. 7 shows median GFAP levels in patients on extracorporeal membrane oxygenation (ECMO) (n=22).

FIG. 8 shows GFAP levels on post-natal day 1 in neonates with (n=27) and without (n=76) neurologic birth injury. Values are mean + SD.

FIG. 9 presents ROC curves of GFAP vs. meconium aspiration as predictors of birth neurologic injury. Top: Logistic regression of neurologic injury related to GFAP > 0.08 ng/ml within 1 day of birth matched for gestational age. Bottom: Meconium stained fluid as a predictor of neurologic injury.

FIG. 10 shows the logistic regression of neonatal serum GFAP at admission to the NICU (Day 0).

FIG. 11 is a graph displaying serum GFAP levels during cardiopulmonary bypass for repair of congenital heart disease, n=20.

FIG. 12 is a graph displaying serial serum GFAP levels during cardiopulmonary bypass for repair of congenital heart disease, n=20.

FIG. 13 is a graph showing GFAP levels during cardiopulmonary bypass for repair of congenital heart disease.

FIG. 14 is table showing demographic and clinical characteristics of the 22 evaluated patients.

FIG. 15 is a table showing individual patient characteristics and outcomes.

FIG. 16 shows peak plasma GFAP concentrations in children on ECMO with and without acute neurologic injury (n=22). Note logarithmic scale on y-axis.

FIG. 17A shows serial plasma GFAP concentrations in children on ECMO with acute neurologic injury (n=7). *X* represents death in the pediatric intensive care unit; *open squares* represent the time of diagnosis of acute neurologic injury closest in time (within 24 hrs) to the last GFAP measurement; *dashed line* marks the 95th percentile of normal controls. Note only six *open squares;* one patient had a diagnostic head ultrasound 48 hrs after the only GFAP measurement (GFAP measurement 24 hrs before diagnosis is missing). FIG. 17B shows serial plasma GFAP concentrations in children on ECMO without acute neurologic injury (n=15). *X* represents death in the pediatric intensive care unit; *dashed line* marks the 95th percentile of normal controls. Note the different scales in *A* and *B*.

FIG. 18 is a table summarizing the maternal and neonatal characteristics of the study population.

FIG. 19 displays box plots of GFAP levels (ng/ml) for control vs. cooled neonates. The number under each box is the number of samples available for analysis. * indicates *P*<.05 compared with controls.

FIG. 20 is a table showing the characteristics for neonates treating with cooling.

FIG. 21 displays box plots of GFAP levels (ng/ml) for cooled neonates compared by results of brain MRI. The number under each box is the number of samples available for analysis. * indicates *P*<.05 compared with neonates with normal MRI.

FIG. 22 is table showing GFAP levels and neurological outcomes.

FIG. 23 shows the spectra ion table for one of 16 unique TSP-1 peptides identified. The Log (e) probability was $10^{-8.6}$. This peptide was found in 10 of 15 SITT patients.

FIG. 24 shows the spectra ion table for one of 3 unique SELL peptides identified. The Log (e) probability was $10^{-4.3}$. This peptide was found in 13 of 15 SITT patients.

FIG. 25 presents TSP-1 concentrations in age-matched (5-14 year old) normal and SCD children.

FIG. 26 shows plasma TSP-1 concentrations in steady-state sickle cell disease (5-14 years old). TSP-1 content is TSP-1 concentration/1×10^6 platelets. *=P< 0.013 or 0.039 SCI vs. non-SCI.

FIG. 27 shows SELL concentration (mean) by ELISA in SCI and non-SCI SCD children and age and race matched controls (top). The same data is shown in bar graph format (middle) and spot graph format with mean (bottom).

FIG. 28 is a gel showing the minimum amount of neurogranin aptamers needed to for detection of neurogranin protein.

FIG. 29 shows the results of a pull-down assay using the neurogranin aptamers.

FIG. 30 displays the signals of neurogranin signature peptide and labeled standard peptide using an ABI Sciex Qtrap 4000 triple quadrapole mass spectrometer.

FIG. 31 shows His-NRGN on PAGE gel after coomassie staining. The predicted molecular weight of His-NRGN is 8.5 Kd.

FIG. 32 shows the standard curve of the direct ELISA for recombinant NRGN using mouse monoclonal antibody 30.5.2. The concentration range is 0.002-10 ng/ml.

FIG. 33 shows the standard curve of the direct ELISA for recombinant NRGN using an anti-human monoclonal antibody to neurogranin. The concentration range is 75 ng/ml.

FIG. 34 shows neurogranin and GFAP levels from patients undergoing cardiopulmonary bypass for surgical repair of congenital heart disease.

DETAILED DESCRIPTION OF THE INVENTION

It is understood that the present invention is not limited to the particular methods and components, etc., described herein, as these may vary. It is also to be understood that the terminology used herein is used for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention. It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include the plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to a "protein" is a reference to one or more proteins, and includes equivalents thereof known to those skilled in the art and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Specific methods, devices, and materials are described, although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention.

All publications cited herein are hereby incorporated by reference including all journal articles, books, manuals, published patent applications, and issued patents. In addition, the meaning of certain terms and phrases employed in the specification, examples, and appended claims are provided. The definitions are not meant to be limiting in nature and

5   serve to provide a clearer understanding of certain aspects of the present invention.

I.   Definitions

The term "brain injury" refers to a condition in which the brain is damaged by injury caused by an event. As used herein, an "injury" is an alteration in cellular or molecular integrity, activity, level, robustness, state, or other alteration that is traceable to an event. For

10   example, an injury includes a physical, mechanical, chemical, biological, functional, infectious, or other modulator of cellular or molecular characteristics. An event can include a physical trauma such as an impact (percussive) or a biological abnormality such as a stroke resulting from either blockade or leakage of a blood vessel. An event is optionally an infection by an infectious agent. A person of skill in the art recognizes numerous equivalent

15   events that are encompassed by the terms injury or event.

More specifically, the term "brain injury" refers to a condition that results in central nervous system damage, irrespective of its pathophysiological basis. Among the most frequent origins of a "brain injury" are stroke and traumatic brain injury (TBI). A "stroke" is classified into hemorrhagic and non-hemorrhagic. Examples of hemorrhagic stroke include

20   cerebral hemorrhage, subarachnoid hemorrhage, and intracranial hemorrhage secondary to cerebral arterial malformation, while examples of non-hemorrhagic stroke include cerebral infarction.

The term "traumatic brain injury" or "TBI" refer to traumatic injuries to the brain which occur when physical trauma causes brain damage. For example, TBI can result from a

25   closed head injury or a penetrating head injury. A "non-traumatic brain injury" refers to brain injuries that do not involve ischemia or external mechanical force (e.g., stroke, Alzheimer's disease, Parkinson's disease, Huntington's disease, multiple sclerosis, amyotrophic lateral sclerosis, brain hemorrhage, brain infections, brain tumor, among others).

The term "brain injury" also refers to subclinical brain injury, spinal cord injury, and

30   anoxic-ischemic brain injury. The term "subclinical brain injury" (SCI) refers to brain injury without overt clinical evidence of brain injury. A lack of clinical evidence of brain injury when brain injury actually exists could result from degree of injury, type of injury, level of consciousness, medications particularly sedation and anesthesia.

The "spinal cord injury" refers to a condition in which the spinal cord receives compression/detrition due to a vertebral fracture or dislocation to cause dysfunction. As used herein, the term "anoxic-ischemic brain injury" refers to deprivation of oxygen supply to brain tissue resulting in compromised brain function and includes cerebral hypoxia. For

5   example, anoxic-ischemic brain injury includes focal cerebral ischemia, global cerebral ischemia, hypoxic hypoxia (i.e., limited oxygen in the environment causes reduced brain function, such as with divers, aviators, mountain climbers, and fire fighters, all of whom are at risk for this kind of cerebral hypoxia), obstructions in the lungs (e.g., hypoxia resulting from choking, strangulation, the crushing of the windpipe)..

10   As used herein, the term "comparing" refers to making an assessment of how the proportion, level or cellular localization of one or more biomarkers in a sample from a patient relates to the proportion, level or cellular localization of the corresponding one or more biomarkers in a standard or control sample. For example, "comparing" may refer to assessing whether the proportion, level, or cellular localization of one or more biomarkers in

15   a sample from a patient is the same as, more or less than, or different from the proportion, level, or cellular localization of the corresponding one or more biomarkers in standard or control sample. More specifically, the term may refer to assessing whether the proportion, level, or cellular localization of one or more biomarkers in a sample from a patient is the same as, more or less than, different from or otherwise corresponds (or not) to the proportion,

20   level, or cellular localization of predefined biomarker levels that correspond to, for example, a patient having subclinical brain injury (SCI), not having SCI, is responding to treatment for SCI, is not responding to treatment for SCI, is/is not likely to respond to a particular SCI treatment, or having /not having another disease or condition. In a specific embodiment, the term "comparing" refers to assessing whether the level of one or more biomarkers of the

25   present invention in a sample from a patient is the same as, more or less than, different from other otherwise correspond (or not) to levels of the same biomarkers in a control sample (e.g., predefined levels that correlate to uninfected individuals, standard SCI levels, etc.).

As used herein, the terms "indicates" or "correlates" (or "indicating" or "correlating," or "indication" or "correlation," depending on the context) in reference to a parameter, e.g., a

30   modulated proportion, level, or cellular localization in a sample from a patient, may mean that the patient has SCI. In specific embodiments, the parameter may comprise the level of one or more biomarkers of the present invention. A particular set or pattern of the amounts of one or more biomarkers may indicate that a patient has SCI (i.e., correlates to a patient having SCI). In other embodiments, a particular set or pattern of the amounts of one or more

biomarkers may be correlated to a patient being unaffected (i.e., indicates a patient does not have SCI). In certain embodiments, "indicating," or "correlating," as used according to the present invention, may be by any linear or non-linear method of quantifying the relationship between levels of biomarkers to a standard, control or comparative value for the assessment

5   of the diagnosis, prediction of SCI or SCI progression, assessment of efficacy of clinical treatment, identification of a patient that may respond to a particular treatment regime or pharmaceutical agent, monitoring of the progress of treatment, and in the context of a screening assay, for the identification of an anti-SCI therapeutic.

The terms "patient," "individual," or "subject" are used interchangeably herein, and

10   refer to a mammal, particularly, a human. The patient may have mild, intermediate or severe disease. The patient may be treatment naïve, responding to any form of treatment, or refractory. The patient may be an individual in need of treatment or in need of diagnosis based on particular symptoms or family history. In some cases, the terms may refer to treatment in experimental animals, in veterinary application, and in the development of

15   animal models for disease, including, but not limited to, rodents including mice, rats, and hamsters; and primates.

The terms "measuring" and "determining" are used interchangeably throughout, and refer to methods which include obtaining a patient sample and/or detecting the level of a biomarker(s) in a sample. In one embodiment, the terms refer to obtaining a patient sample

20   and detecting the level of one or more biomarkers in the sample. In another embodiment, the terms "measuring" and "determining" mean detecting the level of one or more biomarkers in a patient sample. Measuring can be accomplished by methods known in the art and those further described herein. The term "measuring" is also used interchangeably throughout with the term "detecting."

25   The terms "sample," "patient sample," "biological sample," and the like, encompass a variety of sample types obtained from a patient, individual, or subject and can be used in a diagnostic or monitoring assay. The patient sample may be obtained from a healthy subject, a diseased patient or a patient having associated symptoms of SCI. Moreover, a sample obtained from a patient can be divided and only a portion may be used for diagnosis. Further,

30   the sample, or a portion thereof, can be stored under conditions to maintain sample for later analysis. The definition specifically encompasses blood and other liquid samples of biological origin (including, but not limited to, peripheral blood, serum, plasma, cerebrospinal fluid, urine, saliva, stool and synovial fluid), solid tissue samples such as a biopsy specimen or tissue cultures or cells derived therefrom and the progeny thereof. In a

specific embodiment, a sample comprises a blood sample. In another embodiment, a serum sample is used. The definition also includes samples that have been manipulated in any way after their procurement, such as by centrifugation, filtration, precipitation, dialysis, chromatography, treatment with reagents, washed, or enriched for certain cell populations.

5   The terms further encompass a clinical sample, and also include cells in culture, cell supernatants, tissue samples, organs, and the like. Samples may also comprise fresh-frozen and/or formalin-fixed, paraffin-embedded tissue blocks, such as blocks prepared from clinical or pathological biopsies, prepared for pathological analysis or study by immunohistochemistry.

10   Various methodologies of the instant invention include a step that involves comparing a value, level, feature, characteristic, property, etc. to a "suitable control," referred to interchangeably herein as an "appropriate control" or a "control sample." A "suitable control," "appropriate control" or a "control sample" is any control or standard familiar to one of ordinary skill in the art useful for comparison purposes. In one embodiment, a

15   "suitable control" or "appropriate control" is a value, level, feature, characteristic, property, etc., determined in a cell, organ, or patient, e.g., a control or normal cell, organ, or patient, exhibiting, for example, normal traits. For example, the biomarkers of the present invention may be assayed for levels in a sample from an unaffected individual (UI) or a normal control individual (NC) (both terms are used interchangeably herein). In another embodiment, a

20   "suitable control" or "appropriate control" is a value, level, feature, characteristic, property, etc. determined prior to performing a therapy (e.g., an SCI treatment) on a patient. In yet another embodiment, a transcription rate, mRNA level, translation rate, protein level, biological activity, cellular characteristic or property, genotype, phenotype, etc., can be determined prior to, during, or after administering a therapy into a cell, organ, or patient. In a

25   further embodiment, a "suitable control" or "appropriate control" is a predefined value, level, feature, characteristic, property, etc. A "suitable control" can be a profile or pattern of levels of one or more biomarkers of the present invention that correlates to SCI, to which a patient sample can be compared. The patient sample can also be compared to a negative control, i.e., a profile that correlates to not having SCI.

30 II.   Detection of Brain Injury Biomarkers

        A.   Detection by Mass Spectrometry

        In one aspect, the biomarkers of the present invention may be detected by mass spectrometry, a method that employs a mass spectrometer to detect gas phase ions. Examples of mass spectrometers are time-of-flight, magnetic sector, quadrupole filter, ion trap, ion

cyclotron resonance, electrostatic sector analyzer, hybrids or combinations of the foregoing, and the like. In a specific embodiment, the mass spectrometric method comprises matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF MS or MALDI-TOF). In another embodiment, method comprises MALDI-TOF tandem mass spectrometry (MALDI-

5   TOF MS/MS). In yet another embodiment, mass spectrometry can be combined with another appropriate method(s) as may be contemplated by one of ordinary skill in the art. For example, MALDI-TOF can be utilized with trypsin digestion and tandem mass spectrometry as described herein. In another embodiment, the mass spectrometric technique is multiple reaction monitoring (MRM) or quantitative MRM.

10   In an alternative embodiment, the mass spectrometric technique comprises surface enhanced laser desorption and ionization or "SELDI," as described, for example, in U.S. Patents No. 6,225,047 and No. 5,719,060. Briefly, SELDI refers to a method of desorption/ionization gas phase ion spectrometry (e.g. mass spectrometry) in which an analyte (here, one or more of the biomarkers) is captured on the surface of a SELDI mass

15   spectrometry probe. There are several versions of SELDI that may be utilized including, but not limited to, Affinity Capture Mass Spectrometry (also called Surface-Enhanced Affinity Capture (SEAC)), and Surface-Enhanced Neat Desorption (SEND) which involves the use of probes comprising energy absorbing molecules that are chemically bound to the probe surface (SEND probe). Another SELDI method is called Surface-Enhanced Photolabile

20   Attachment and Release (SEPAR), which involves the use of probes having moieties attached to the surface that can covalently bind an analyte, and then release the analyte through breaking a photolabile bond in the moiety after exposure to light, e.g., to laser light (see, U.S. Patent No. 5,719,060). SEPAR and other forms of SELDI are readily adapted to detecting a biomarker or biomarker panel, pursuant to the present invention.

25   In another mass spectrometry method, the biomarkers can be first captured on a chromatographic resin having chromatographic properties that bind the biomarkers. For example, one could capture the biomarkers on a cation exchange resin, such as CM Ceramic HyperD F resin, wash the resin, elute the biomarkers and detect by MALDI. Alternatively, this method could be preceded by fractionating the sample on an anion exchange resin before

30   application to the cation exchange resin. In another alternative, one could fractionate on an anion exchange resin and detect by MALDI directly. In yet another method, one could capture the biomarkers on an immuno-chromatographic resin that comprises antibodies that bind the biomarkers, wash the resin to remove unbound material, elute the biomarkers from the resin and detect the eluted biomarkers by MALDI or by SELDI.

B.   Detection by Immunoassay

In other embodiments, the biomarkers of the present invention can be detected and/or measured by immunoassay. Immunoassay requires biospecific capture reagents, such as antibodies, to capture the biomarkers. Many antibodies are available commercially.

5   Antibodies also can be produced by methods well known in the art, e.g., by immunizing animals with the biomarkers. Biomarkers can be isolated from samples based on their binding characteristics. Alternatively, if the amino acid sequence of a polypeptide biomarker is known, the polypeptide can be synthesized and used to generate antibodies by methods well-known in the art.

10   The present invention contemplates traditional immunoassays including, for example, sandwich immunoassays including ELISA or fluorescence-based immunoassays, immunoblots, Western Blots (WB), as well as other enzyme immunoassays. Nephelometry is an assay performed in liquid phase, in which antibodies are in solution. Binding of the antigen to the antibody results in changes in absorbance, which is measured. In a SELDI-

15   based immunoassay, a biospecific capture reagent for the biomarker is attached to the surface of an MS probe, such as a pre-activated protein chip array. The biomarker is then specifically captured on the biochip through this reagent, and the captured biomarker is detected by mass spectrometry.

Although antibodies are useful because of their extensive characterization, any other

20   suitable agent (e.g., a peptide, an aptamer, or a small organic molecule) that specifically binds a biomarker of the present invention is optionally used in place of the antibody in the above described immunoassays. For example, an aptamer that specifically binds all neurogranin and/or one or more of its breakdown products might be used. Aptamers are nucleic acid-based molecules that bind specific ligands. Methods for making aptamers with a particular

25   binding specificity are known as detailed in U.S. Patents No. 5,475,096; No. 5,670,637; No. 5,696,249; No. 5,270,163; No. 5,707,796; No. 5,595,877; No. 5,660,985; No. 5,567,588; No. 5,683,867; No. 5,637,459; and No. 6,011,020.

C.   Detection by Electrochemicaluminescent Assay

In several embodiments, the biomarker biomarkers of the present invention may be

30   detected by means of an electrochemicaluminescent assay developed by Meso Scale Discovery (Gaithersrburg, MD). Electrochemiluminescence detection uses labels that emit light when electrochemically stimulated. Background signals are minimal because the stimulation mechanism (electricity) is decoupled from the signal (light). Labels are stable, non-radioactive and offer a choice of convenient coupling chemistries. They emit light at

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~620 nm, eliminating problems with color quenching. *See* U.S. Patents No. 7,497,997; No. 7,491,540; No. 7,288,410; No. 7,036,946; No. 7,052,861; No. 6,977,722; No. 6,919,173; No. 6,673,533; No. 6,413,783; No. 6,362,011; No. 6,319,670; No. 6,207,369; No. 6,140,045; No. 6,090,545; and No. 5,866,434. *See also* U.S. Patent Applications Publication No.

5   2009/0170121; No. 2009/006339; No. 2009/0065357; No. 2006/0172340; No. 2006/0019319; No. 2005/0142033; No. 2005/0052646; No. 2004/0022677; No. 2003/0124572; No. 2003/0113713; No. 2003/0003460; No. 2002/0137234; No. 2002/0086335; and No. 2001/0021534.

D.   Other Methods for Detecting Biomarkers

10   The biomarkers of the present invention can be detected by other suitable methods. Detection paradigms that can be employed to this end include optical methods, electrochemical methods (voltametry and amperometry techniques), atomic force microscopy, and radio frequency methods, e.g., multipolar resonance spectroscopy. Illustrative of optical methods, in addition to microscopy, both confocal and non-confocal,

15   are detection of fluorescence, luminescence, chemiluminescence, absorbance, reflectance, transmittance, and birefringence or refractive index (e.g., surface plasmon resonance, ellipsometry, a resonant mirror method, a grating coupler waveguide method or interferometry).

Furthermore, a sample may also be analyzed by means of a biochip. Biochips

20   generally comprise solid substrates and have a generally planar surface, to which a capture reagent (also called an adsorbent or affinity reagent) is attached. Frequently, the surface of a biochip comprises a plurality of addressable locations, each of which has the capture reagent bound there. Protein biochips are biochips adapted for the capture of polypeptides. Many protein biochips are described in the art. These include, for example, protein biochips

25   produced by Ciphergen Biosystems, Inc. (Fremont, CA.), Invitrogen Corp. (Carlsbad, CA), Affymetrix, Inc. (Fremong, CA), Zyomyx (Hayward, CA), R&D Systems, Inc. (Minneapolis, MN), Biacore (Uppsala, Sweden) and Procognia (Berkshire, UK). Examples of such protein biochips are described in the following patents or published patent applications: U.S. Patent No. 6,537,749; U.S. Patent No. 6,329,209; U.S. Patent No. 6,225,047; U.S. Patent No.

30   5,242,828; PCT International Publication No. WO 00/56934; and PCT International Publication No. WO 03/048768.

III.   Determination of a Patient's Brain Injury Status

The present invention relates to the use of biomarkers to diagnose brain injury. More specifically, the biomarkers of the present invention can be used in diagnostic tests to

determine, qualify, and/or assess brain injury status, for example, to diagnose brain injury, in an individual, subject or patient. In particular embodiments, brain injury status can include determining a patient's subclinical brain injury status or SCI status, for example, to diagnose SCI, in an individual, subject or patient. More specifically, the biomarkers to be detected in

5   diagnosing brain injury (e.g., SCI or overt brain injury) include, but are not limited to, ASTN1, BAI3, CNDP1, ERMIN, GFAP, GRM3, KLH32, MAGE2, NRG3, NRGN, OMG, SLC39A12, RTN1, and MT3. Other biomarkers known in the relevant art may be used in combination with the biomarkers described herein including, but not limited to, SBDP150, SBDP150i, NSE, S1OOβ, MAP2, MAPI, MAP3, MAP4, MAP5, MBP, Tau, NF-L, NF-M, NF-H,

10   UCH-L1, NSE, NeuN, CNPase, α-internexin, CB-I, CB-2; ICAM, VAM, NCAM, NL-CAM, AL-CAM, C-CAM; synaptotagmin, synaptophysin, synapsin, SNAP; CRMP-2, CRMP-I, CRMP-3, CRMP-4 iNOS, and βlll-tubulin.

A.   Biomarker Panels

The biomarkers of the present invention can be used in diagnostic tests to assess,

15   determine, and/or qualify (used interchangeably herein) brain injury status in a patient. The phrase "brain injury status" includes any distinguishable manifestation of the condition, including not having brain injury. For example, brain injury status includes, without limitation, the presence or absence of brain injury in a patient, the risk of developing brain injury, the stage or severity of brain injury, the progress of brain injury (e.g., progress of

20   brain injury over time) and the effectiveness or response to treatment of brain injury (e.g., clinical follow up and surveillance of brain injury after treatment). Based on this status, further procedures may be indicated, including additional diagnostic tests or therapeutic procedures or regimens.

The power of a diagnostic test to correctly predict status is commonly measured as the

25   sensitivity of the assay, the specificity of the assay or the area under a receiver operated characteristic ("ROC") curve. Sensitivity is the percentage of true positives that are predicted by a test to be positive, while specificity is the percentage of true negatives that are predicted by a test to be negative. An ROC curve provides the sensitivity of a test as a function of 1-specificity. The greater the area under the ROC curve, the more powerful the predictive

30   value of the test. Other useful measures of the utility of a test are positive predictive value and negative predictive value. Positive predictive value is the percentage of people who test positive that are actually positive. Negative predictive value is the percentage of people who test negative that are actually negative.

In particular embodiments, the biomarker panels of the present invention may show a statistical difference in different SCI statuses of at least $p<0.05$, $p<10^{-2}$, $p<10^{-3}$, $p<10^{-4}$ or $p<10^{-5}$. Diagnostic tests that use these biomarkers may show an ROC of at least 0.6, at least about 0.7, at least about 0.8, or at least about 0.9.

5   The biomarkers are differentially present in UI (NC or non- brain injury) and brain injury, and, therefore, are useful in aiding in the determination of brain injury status. In certain embodiments, the biomarkers are measured in a patient sample using the methods described herein and compared, for example, to predefined biomarker levels and correlated to brain injury status. In particular embodiments, the measurement(s) may then be compared

10   with a relevant diagnostic amount(s), cut-off(s), or multivariate model scores that distinguish a positive brain injury status from a negative brain injury status. The diagnostic amount(s) represents a measured amount of a biomarker(s) above which or below which a patient is classified as having a particular brain injury status. For example, if the biomarker(s) is/are up-regulated compared to normal during brain injury, then a measured amount(s) above the

15   diagnostic cutoff(s) provides a diagnosis of brain injury. Alternatively, if the biomarker(s) is/are down-regulated during brain injury, then a measured amount(s) at or below the diagnostic cutoff(s) provides a diagnosis of non-brain injury. As is well understood in the art, by adjusting the particular diagnostic cut-off(s) used in an assay, one can increase sensitivity or specificity of the diagnostic assay depending on the preference of the diagnostician. In

20   particular embodiments, the particular diagnostic cut-off can be determined, for example, by measuring the amount of biomarkers in a statistically significant number of samples from patients with the different brain injury statuses, and drawing the cut-off to suit the desired levels of specificity and sensitivity.

Indeed, as the skilled artisan will appreciate there are many ways to use the

25   measurements of two or more biomarkers in order to improve the diagnostic question under investigation. In a quite simple, but nonetheless often effective approach, a positive result is assumed if a sample is positive for at least one of the markers investigated.

Furthermore, in certain embodiments, the values measured for markers of a biomarker panel are mathematically combined and the combined value is correlated to the underlying

30   diagnostic question. Biomarker values may be combined by any appropriate state of the art mathematical method. Well-known mathematical methods for correlating a marker combination to a disease status employ methods like discriminant analysis (DA) (e.g., linear-, quadratic-, regularized-DA), Discriminant Functional Analysis (DFA), Kernel Methods (e.g., SVM), Multidimensional Scaling (MDS), Nonparametric Methods (e.g., k-Nearest-Neighbor

Classifiers), PLS (Partial Least Squares), Tree-Based Methods (e.g., Logic Regression, CART, Random Forest Methods, Boosting/Bagging Methods), Generalized Linear Models (e.g., Logistic Regression), Principal Components based Methods (e.g., SIMCA), Generalized Additive Models, Fuzzy Logic based Methods, Neural Networks and Genetic Algorithms based Methods. The skilled artisan will have no problem in selecting an appropriate method to evaluate a biomarker combination of the present invention. In one embodiment, the method used in a correlating a biomarker combination of the present invention, e.g. to diagnose brain injury, is selected from DA (e.g., Linear-, Quadratic-, Regularized Discriminant Analysis), DFA, Kernel Methods (e.g., SVM), MDS, Nonparametric Methods (e.g., k-Nearest-Neighbor Classifiers), PLS (Partial Least Squares), Tree-Based Methods (e.g., Logic Regression, CART, Random Forest Methods, Boosting Methods), or Generalized Linear Models (e.g., Logistic Regression), and Principal Components Analysis. Details relating to these statistical methods are found in the following references: Ruczinski et al.,12 J. OF COMPUTATIONAL AND GRAPHICAL STATISTICS 475-511 (2003); Friedman, J. H., 84 J. OF THE AMERICAN STATISTICAL ASSOCIATION 165-75 (1989); Hastie, Trevor, Tibshirani, Robert, Friedman, Jerome, The Elements of Statistical Learning, Springer Series in Statistics (2001); Breiman, L., Friedman, J. H., Olshen, R. A., Stone, C. J. Classification and regression trees, California: Wadsworth (1984); Breiman, L., 45 MACHINE LEARNING 5-32 (2001); Pepe, M. S., The Statistical Evaluation of Medical Tests for Classification and Prediction, Oxford Statistical Science Series, 28 (2003); and Duda, R. O., Hart, P. E., Stork, D. G., Pattern Classification, Wiley Interscience, 2nd Edition (2001).

B.   Determining Risk of Developing Brain Injury

In a specific embodiment, the present invention provides methods for determining the risk of developing brain injury in a patient. Biomarker percentages, amounts or patterns are characteristic of various risk states, e.g., high, medium or low. The risk of developing brain injury is determined by measuring the relevant biomarkers and then either submitting them to a classification algorithm or comparing them with a reference amount, i.e., a predefined level or pattern of biomarkers that is associated with the particular risk level.

C.   Determining Brain Injury Severity

In another embodiment, the present invention provides methods for determining the severity of brain injury in a patient. Each grade or stage of brain injury likely has a characteristic level of a biomarker or relative levels of a set of biomarkers (a pattern). The severity of brain injury is determined by measuring the relevant biomarkers and then either

submitting them to a classification algorithm or comparing them with a reference amount, i.e., a predefined level or pattern of biomarkers that is associated with the particular stage.

D.   Determining Brain Injury Prognosis

In one embodiment, the present invention provides methods for determining the

5   course of brain injury in a patient. brain injury course refers to changes in brain injury status over time, including brain injury progression (worsening) and brain injury regression (improvement). Over time, the amount or relative amount (e.g., the pattern) of the biomarkers changes. For example, biomarker "X" may be increased with brain injury, while biomarker "Y" may be decreased with brain injury. Therefore, the trend of these biomarkers,

10   either increased or decreased over time toward brain injury or non-brain injury indicates the course of the condition. Accordingly, this method involves measuring the level of one or more biomarkers in a patient at least two different time points, e.g., a first time and a second time, and comparing the change, if any. The course of brain injury is determined based on these comparisons.

15   E.   Patient Management

In certain embodiments of the methods of qualifying brain injury status, the methods further comprise managing patient treatment based on the status. Such management includes the actions of the physician or clinician subsequent to determining brain injury status. For example, if a physician makes a diagnosis of brain injury, then a certain regime of monitoring

20   would follow. An assessment of the course of brain injury using the methods of the present invention may then require a certain brain injury therapy regimen. Alternatively, a diagnosis of non-brain injury might be followed with further testing to determine a specific disease that the patient might be suffering from. Also, further tests may be called for if the diagnostic test gives an inconclusive result on brain injury status.

25   F.   Determining Therapeutic Efficacy of Pharmaceutical Drug

In another embodiment, the present invention provides methods for determining the therapeutic efficacy of a pharmaceutical drug. These methods are useful in performing clinical trials of the drug, as well as monitoring the progress of a patient on the drug. Therapy or clinical trials involve administering the drug in a particular regimen. The regimen

30   may involve a single dose of the drug or multiple doses of the drug over time. The doctor or clinical researcher monitors the effect of the drug on the patient or subject over the course of administration. If the drug has a pharmacological impact on the condition, the amounts or relative amounts (e.g., the pattern or profile) of one or more of the biomarkers of the present invention may change toward a non-brain injury profile. Therefore, one can follow the

course of one or more biomarkers in the patient during the course of treatment. Accordingly, this method involves measuring one or more biomarkers in a patient receiving drug therapy, and correlating the biomarker levels with the brain injury status of the patient (e.g., by comparison to predefined levels of the biomarkers that correspond to different brain injury

5   statuses). One embodiment of this method involves determining the levels of one or more biomarkers at at least two different time points during a course of drug therapy, e.g., a first time and a second time, and comparing the change in levels of the biomarkers, if any. For example, the levels of one or more biomarkers can be measured before and after drug administration or at two different time points during drug administration. The effect of

10   therapy is determined based on these comparisons. If a treatment is effective, then the one or more biomarkers will trend toward normal, while if treatment is ineffective, the one or more biomarkers will trend toward brain injury indications.

G.   Generation of Classification Algorithms for Qualifying Brain Injury Status

In some embodiments, data that are generated using samples such as "known

15   samples" can then be used to "train" a classification model. A "known sample" is a sample that has been pre-classified. The data that are used to form the classification model can be referred to as a "training data set." The training data set that is used to form the classification model may comprise raw data or pre-processed data. Once trained, the classification model can recognize patterns in data generated using unknown samples. The classification model

20   can then be used to classify the unknown samples into classes. This can be useful, for example, in predicting whether or not a particular biological sample is associated with a certain biological condition (e.g., diseased versus non-diseased).

Classification models can be formed using any suitable statistical classification or learning method that attempts to segregate bodies of data into classes based on objective

25   parameters present in the data. Classification methods may be either supervised or unsupervised. Examples of supervised and unsupervised classification processes are described in Jain, "Statistical Pattern Recognition: A Review", IEEE Transactions on Pattern Analysis and Machine Intelligence, Vol. 22, No. 1, January 2000, the teachings of which are incorporated by reference.

30   In supervised classification, training data containing examples of known categories are presented to a learning mechanism, which learns one or more sets of relationships that define each of the known classes. New data may then be applied to the learning mechanism, which then classifies the new data using the learned relationships. Examples of supervised classification processes include linear regression processes (e.g., multiple linear regression

(MLR), partial least squares (PLS) regression and principal components regression (PCR)), binary decision trees (e.g., recursive partitioning processes such as CART), artificial neural networks such as back propagation networks, discriminant analyses (e.g., Bayesian classifier or Fischer analysis), logistic classifiers, and support vector classifiers (support vector machines).

Another supervised classification method is a recursive partitioning process. Recursive partitioning processes use recursive partitioning trees to classify data derived from unknown samples. Further details about recursive partitioning processes are provided in U.S. Patent Application No. 2002 0138208 A1 to Paulse et al., "Method for analyzing mass spectra."

In other embodiments, the classification models that are created can be formed using unsupervised learning methods. Unsupervised classification attempts to learn classifications based on similarities in the training data set, without pre-classifying the spectra from which the training data set was derived. Unsupervised learning methods include cluster analyses. A cluster analysis attempts to divide the data into "clusters" or groups that ideally should have members that are very similar to each other, and very dissimilar to members of other clusters. Similarity is then measured using some distance metric, which measures the distance between data items, and clusters together data items that are closer to each other. Clustering techniques include the MacQueen's K-means algorithm and the Kohonen's Self-Organizing Map algorithm.

Learning algorithms asserted for use in classifying biological information are described, for example, in PCT International Publication No. WO 01/31580 (Barnhill et al., "Methods and devices for identifying patterns in biological systems and methods of use thereof"), U.S. Patent Application Publication No. 2002/0193950 (Gavin et al. "Method or analyzing mass spectra"), U.S. Patent Application Publication No. 2003/0004402 (Hitt et al., "Process for discriminating between biological states based on hidden patterns from biological data"), and U.S. Patent Application Publication No. 2003/0055615 (Zhang and Zhang, "Systems and methods for processing biological expression data").

The classification models can be formed on and used on any suitable digital computer. Suitable digital computers include micro, mini, or large computers using any standard or specialized operating system, such as a Unix, Windows® or Linux™ based operating system. In embodiments utilizing a mass spectrometer, the digital computer that is used may be physically separate from the mass spectrometer that is used to create the spectra of interest, or it may be coupled to the mass spectrometer.

The training data set and the classification models according to embodiments of the invention can be embodied by computer code that is executed or used by a digital computer. The computer code can be stored on any suitable computer readable media including optical or magnetic disks, sticks, tapes, etc., and can be written in any suitable computer

5   programming language including R, C, C++, visual basic, etc.

The learning algorithms described above are useful both for developing classification algorithms for the biomarkers already discovered, and for finding new biomarker biomarkers. The classification algorithms, in turn, form the base for diagnostic tests by providing diagnostic values (e.g., cut-off points) for biomarkers used singly or in combination.

10   H.   Kits for the Detection of Brain Injury Biomarkers

In another aspect, the present invention provides kits for qualifying brain injury status, which kits are used to detect the biomarkers described herein. In a specific embodiment, the kit is provided as an ELISA kit comprising antibodies to the biomarkers of the present invention including, but not limited to, ASTN1, BAI3, CNDP1, ERMIN, GFAP, GRM3,

15   KLH32, MAGE2, NRG3, NRGN, OMG, SLC39A12, RTN1, and MT3.

The ELISA kit may comprise a solid support, such as a chip, microtiter plate (e.g., a 96-well plate), bead, or resin having biomarker capture reagents attached thereon. The kit may further comprise a means for detecting the biomarkers, such as antibodies, and a secondary antibody-signal complex such as horseradish peroxidase (HRP)-conjugated goat

20   anti-rabbit IgG antibody and tetramethyl benzidine (TMB) as a substrate for HRP.

The kit for qualifying brain injury status may be provided as an immuno-chromatography strip comprising a membrane on which the antibodies are immobilized, and a means for detecting, e.g., gold particle bound antibodies, where the membrane, includes NC membrane and PVDF membrane. The kit may comprise a plastic plate on which a sample

25   application pad, gold particle bound antibodies temporally immobilized on a glass fiber filter, a nitrocellulose membrane on which antibody bands and a secondary antibody band are immobilized and an absorbent pad are positioned in a serial manner, so as to keep continuous capillary flow of blood serum.

In certain embodiments, a patient can be diagnosed by adding blood or blood serum

30   from the patient to the kit and detecting the relevant biomarkers conjugated with antibodies, specifically, by a method which comprises the steps of: (i) collecting blood or blood serum from the patient; (ii) separating blood serum from the patient's blood; (iii) adding the blood serum from patient to a diagnostic kit; and, (iv) detecting the biomarkers conjugated with antibodies. In this method, the antibodies are brought into contact with the patient's blood.

If the biomarkers are present in the sample, the antibodies will bind to the sample, or a portion thereof. In other kit and diagnostic embodiments, blood or blood serum need not be collected from the patient (i.e., it is already collected). Moreover, in other embodiments, the sample may comprise a tissue sample or a clinical sample.

5   The kit can also comprise a washing solution or instructions for making a washing solution, in which the combination of the capture reagents and the washing solution allows capture of the biomarkers on the solid support for subsequent detection by, e.g., antibodies or mass spectrometry. In a further embodiment, a kit can comprise instructions for suitable operational parameters in the form of a label or separate insert. For example, the instructions

10   may inform a consumer about how to collect the sample, how to wash the probe or the particular biomarkers to be detected, etc. In yet another embodiment, the kit can comprise one or more containers with biomarker samples, to be used as standard(s) for calibration.

          Without further elaboration, it is believed that one skilled in the art, using the preceding description, can utilize the present invention to the fullest extent. The following

15   examples are illustrative only, and not limiting of the remainder of the disclosure in any way whatsoever.

<div align="center">EXAMPLES</div>

          The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles,

20   devices, and/or methods described and claimed herein are made and evaluated, and are intended to be purely illustrative and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.) but some errors and deviations should be accounted for herein. Unless indicated otherwise, parts are parts by weight, temperature is in degrees

25   Celsius or is at ambient temperature, and pressure is at or near atmospheric. There are numerous variations and combinations of reaction conditions, e.g., component concentrations, desired solvents, solvent mixtures, temperatures, pressures and other reaction ranges and conditions that can be used to optimize the product purity and yield obtained from the described process. Only reasonable and routine experimentation will be required to

30   optimize such process conditions.

<div align="center">Example 1: Discovery of Circulation Biomarkers of Subclinical Brain Injury (SCI)

in Children with Sickle Cell Disease (SCD)</div>

          Children with SCD and SCI have significant learning defects and are at increased risk for stroke. Early detection of SCI would allow institution of therapies to prevent further brain

<div align="center">23</div>

injury and neurocognitive defects, including loss of I.Q. By definition, SCI patients have MRI and pathologic evidence of brain injury consistent with microinfarction. Therefore, it is likely that unique circulating biomarkers exist to identify patients with SCI as has been shown in non-SCD patients with overt stroke. The present goal of is to use non-biased

5   proteomic techniques to explore clinically paired groups of SCI and non-SCI patient plasma samples to identify lead proteins for validation as circulating biomarkers of SCI.

Study Population. The study population comprised male and female children 6-12 years old with SCD participating in the SIT Trial (24 institutions) having an adjudicated MRI with (SCI group) or without (non-SCI group) evidence of SCI. As part of the SIT Trial, in

10   addition to an MRI and neurologic exam, patients have CBC, Hb/Hct, hemoglobin electrophoresis and other clinical studies determined. At entry into the trial, citrated plasma is prepared, frozen and stored in the Biologic Repository at Johns Hopkins at -70°C. The initial discovery cohort for this Example, as described in Table 1, includes 50 patients with SCI and 50 patients without SCI, all with an Hb < 8.1 g/dL, as this has been shown to be an

15   independent risk factor for SCI.

Depletion of Abundant Proteins. To identify proteins with altered plasma expression in children with SCI, 250 μl aliquots of individual plasma samples from SCI and non-SCI children were affinity depleted using a new generation of the previously available LC10 polyclonal antibody affinity column (IgY high capacity LC10 column, Beckman-Coulter).

20   This new generation column doubles the amount of plasma that can be depleted (from 125 to 250 μl), thereby increasing downstream sensitivity. This new column is coupled to the ProteomeLab PPS Proteome Partitioning System (Beckman-Coulter) for flow through and bound protein collection. For each 250 μl of plasma depleted, 7 ml of flow through protein is collected. Using a 5 ml loop, 400 μg of depleted protein was fractionated by RP HPLC

25   (ProteomeLab PPS Proteome Partitioning System) coupled to a C_{18} non-porous column, using a linear acetonitrile gradient, with 60 fractions collected over 30 minutes.

Identification and Quantification of Small and Low Abundant Plasma Proteins in SCI. To identify the proteins contained in the RP HPLC fractions described above, LC/MS/MS (LTQ Orbitrap) analysis of each RP HPLC fraction was used to develop a non-SCI/SCI

30   plasma protein database. Briefly, the pooled RP HPLC fractions from a single patient were completely dried using a SpeedVac, resuspended in neutralization buffer and trypsin digested overnight. After trypsin digestion, the RP HPLC fractions were analyzed by LC/MS/MS (Thermo LTQ-Orbitrap). RP HPLC fraction of intact proteins before trypsin digestion resulted in fractions contains 50-100 proteins each. To increase protein identification

confidence, each tandem MS spectra RAW file was searched using X! Tandem, OMSSA, and SageN SEQUEST against the IPI protein database (currently vs. 3.6 with full trypsin digestion) and the protein and peptide identification results compiled for each patient in Maspectras and Scaffold. Initial lead proteins were identified based on presence/absence or a $\geq$ 1.5 fold change (increase or decrease) in spectral counts of peptides in the SCI and non-SCI samples.

Brain Injury Protein Identification. Using semi-quantitative spectral count fold change cut off of 1.5, proteins circulating in children with SCI are listed below in Table 1. These peptide identifications have an expectation value of $<10^{-1}$.

Table 1. Brain Injury Protein Biomarkers

Accession No.	Protein Name
P07996	Thrombospondin-1
P14151	L-selectin
P31949	Protein S100-A11
Q9P273	Teneurin-3
P18206	Vinculin
P25815	Protein S100-P
Q86WP2	Vasculin
P50552	Vasodilator-stimulated phosphoprotein
P80511	Protein S100-A12
P41222	Prostaglandin-H2 D-isomerase
Q86V38	Atrophin 1
Q92686	Neurogranin
Q15465	Sonic hedgehog protein
O43426	Synaptojanin-1
P02775	Platelet basic protein
Q05682	Caldesmon
Q9H461	Frizzled-8
P06703	Protein S100-A6
P62158	Calmodulin
P80723	Brain acid soluble protein 1
Q9NQC3	Reticulon-4
Q9Y5Y7	Lymphatic vessel endothelial hyaluronic acid receptor 1
P36955	Pigment epithelium-derived factor
P06702	Protein S100-A9
Q8TBZ2	MYCBP-associated protein
A6NIX2	Wilms tumor protein 1-interacting protein
A0AVI2	Fer-1-like protein 5
O00151	PDZ and LIM domain protein 1
O95744	Postmeiotic segregation increased 2-like protein 2
Q68D06	Schlafen family member 13

Accession No.	Protein Name
P40121	Macrophage-capping protein
Q6UXX5	Inter-alpha-trypsin inhibitor heavy chain H5-like protein
P02788	Lactotransferrin
Q5JNX2	Complement component 4A (Rodgers blood group) (Uncharacterized protein ENSP00000372815)
Q68CR7	Leucine-rich repeat-containing protein 66
A2BHY4	Complement component C4B (Childo blood group)
P17097	Zinc finger protein 7
P19652	Alpha-1-acid glycoprotein 2
Q5VT06	Centrosome-associated protein 350
Q7L211	Abhydrolase domain-containing protein 13
Q9P1Z9	Uncharacterized protein KIAA1529
P98073	Enteropeptidase
Q9P242	Uncharacterized protein KIAA1486
Q7Z5P9	Mucin-19
A6NII9	Putative uncharacterized protein ENSP00000351043 (Family with sequence similarity 19 (Chemokine (C-C motif)-like), member A5, isoform CRA_a) (Putative uncharacterized protein ENSP00000336812)
A8MSL6	Putative uncharacterized protein APOA4
Q7Z7G8	Vacuolar protein sorting-associated protein 13B
P02750	Leucine-rich alpha-2-glycoprotein
P10720	Platelet factor 4 variant
IPI00896413.1	INTER-ALPHA (GLOBULIN) INHIBITOR H4 ISOFORM 2 PRECURSOR.
P15924	Desmoplakin
Q9UKV3	Apoptotic chromatin condensation inducer in the nucleus
O15417	Trinucleotide repeat-containing gene 18 protein
Q06830	Peroxiredoxin-1
Q14766	Latent-transforming growth factor beta-binding protein, isoform 1L
Q49AS2	ELK2, member of ETS oncogene family, pseudogene 1
P55042	GTP-binding protein RAD
P10599	Thioredoxin
P35579	Myosin-9
Q9NVN8	Guanine nucleotide-binding protein-like 3-like protein
Q03828	Homeobox even-skipped homolog protein 2
P16401	Histone H1.5
Q7Z7M0	Multiple epidermal growth factor-like domains 8
Q9NYF0	Dapper homolog 1
Q02747	Guanylin
Q9HCJ5	Zinc finger SWIM domain-containing protein 6
Q8TEH0	FLJ00227 protein
P60323	Nanos homolog 3
Q9P2H0	Uncharacterized protein KIAA1377
Q9Y6X6	Myosin-XVI
O60299	Uncharacterized protein KIAA0552

Accession No.	Protein Name
Q8NDT4	Zinc finger protein 663
Q8NDX1	PH and SEC7 domain-containing protein 4
P02760	Protein AMBP
P20851	C4b-binding protein beta chain
P52209	6-phosphogluconate dehydrogenase, decarboxylating
A6NI80	Uncharacterized protein ENSP00000305613
Q96LW6	cDNA FLJ33157 fis, clone UTERU2000393 (HCG2021706)
Q9NZV6	Methionine-R-sulfoxide reductase B1
P04114	Apolipoprotein B-100
Q8WY24	SNC66 protein
Q5T8A7	Protein KIAA0649
Q68DS3	Putative uncharacterized protein DKFZp686H17246
P02790	Hemopexin
O60907	F-box-like/WD repeat-containing protein TBL1X
P02745	Complement C1q subcomponent subunit A
Q96IY4	Carboxypeptidase B2
Q12947	Forkhead box protein F2
Q13505	Metaxin-1
Q71RA9	PP7706
Q99747	Gamma-soluble NSF attachment protein
P35858	Insulin-like growth factor-binding protein complex acid labile chain
P00736	Complement C1r subcomponent
O15370	SOX-12 protein
P08670	Vimentin
P02746	Complement C1q subcomponent subunit B
Q96CP6	GRAM domain-containing protein 1A
Q92954	Proteoglycan 4
P10412	Histone H1.4
P32119	Peroxiredoxin-2
P06733	Alpha-enolase
Q9UPP5	Uncharacterized protein KIAA1107
Q5T8R8	Uncharacterized protein C9orf66
Q9NY12	H/ACA ribonucleoprotein complex subunit 1
P07360	Complement component C8 gamma chain
P04004	Vitronectin
Q8NCM2	Potassium voltage-gated channel subfamily H member 5
A8K5V8	cDNA FLJ30633 fis,highly similar to Homo sapiens modulator of estrogen induced transcription, transcript variant 1, mRNA
Q9BTL4	Immediate early response gene 2 protein
Q96PD5	N-acetylmuramoyl-L-alanine amidase
IPI00807369.1	IPI00807369.1
IPI00788213.2	IPI00788213.2
B4E2M5	cDNA FLJ58975

Accession No.	Protein Name
IPI00782956.2	IPI00782956.2
IPI00878453.1	IPI00878453.1
P02452	Collagen alpha-1(I) chain
Q9BXB5	Oxysterol-binding protein-related protein 10
IPI00457142.3	SIMILAR TO U2 SMALL NUCLEAR RNA AUXILLARY FACTOR 1
O75528	Transcriptional adapter 3-like
P04196	Histidine-rich glycoprotein
P08123	Collagen alpha-2(I) chain
IPI00867606.2	ISOFORM 2 OF RAS-GEF DOMAIN-CONTAINING FAMILY MEMBER 1B
O95696	Bromodomain-containing protein 1
P49588	Alanyl-tRNA synthetase, cytoplasmic
IPI00794089.1	IPI00794089.1
O75052	Carboxyl-terminal PDZ ligand of neuronal nitric oxide synthase protein
P49748	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial
Q96JQ0	Protocadherin-16
P02654	Apolipoprotein C-I
O94906	Pre-mRNA-processing factor 6
Q96M32	Putative adenylate kinase 7
P36980	Complement factor H-related protein 2
Q9H2Y7	Zinc finger protein 106 homolog
Q68DL7	Uncharacterized protein DKFZp781G0119
IPI00883666.2	IPI00883666.2
Q66PJ3	ADP-ribosylation factor-like protein 6-interacting protein 4
Q9UPA5	Protein bassoon
P60602	Protein MGR2 homolog
Q8N2B8	Putative uncharacterized protein FLJ33534
Q96HY7	Probable 2-oxoglutarate dehydrogenase E1 component DHKTD1, mitochondrial
Q96T92	Insulinoma-associated protein 2
Q9UKK3	Poly [ADP-ribose] polymerase 4
O76042	Uncharacterized protein C3orf51
P02656	Apolipoprotein C-III
P02461	Collagen alpha-1(III) chain
IPI00219910.2	IPI00219910.2
IPI00442185.1	IPI00442185.1
IPI00794089.1	IPI00794089.1
IPI00847731.1	IPI00847731.1

Example 2: Discovery of Circulation Biomarkers of Subclinical Brain Injury (SCI) in Children with Sickle Cell Disease (SCD)

Children with SCD and SCI have significant learning defects and are at increased risk

5   for stroke. Early detection of SCI would allow institution of therapies to prevent further brain

injury and neurocognitive defects, including loss of I.Q. By definition, SCI patients have MRI and pathologic evidence of brain injury consistent with microinfarction. Therefore, it is likely that unique circulating biomarkers exist to identify patients with SCI as has been shown in non-SCD patients with overt stroke. The present goal of is to use non-biased

5   proteomic techniques to explore clinically paired groups of SCI and non-SCI patient plasma samples to identify lead proteins for validation as circulating biomarkers of SCI.

Study Population. The study population comprised male and female children 6-12 years old with SCD participating in the SIT Trial (24 institutions) having an adjudicated MRI with (SCI group) or without (non-SCI group) evidence of SCI. As part of the SIT Trial, in

10 addition to an MRI and neurologic exam, patients have CBC, Hb/Hct, hemoglobin electrophoresis and other clinical studies determined. At entry into the trial, citrated plasma is prepared, frozen and stored in the Biologic Repository at Johns Hopkins at -70°C. The initial discovery cohort for this Example, as described in Table 2, includes 50 patients with SCI and 50 patients without SCI, all with an Hb < 8.1 g/dL, as this has been shown to be an

15 independent risk factor for SCI.

Depletion of Abundant Proteins. To identify proteins with altered plasma expression in children with SCI, 250 μL aliquots of individual plasma samples from SCI and non-SCI children were affinity depleted using a new generation of the previously available LC10 polyclonal antibody affinity column (IgY high capacity LC10 column, Beckman-Coulter).

20 This new generation column doubles the amount of plasma that can be depleted (from 125 to 250 μl), thereby increasing downstream sensitivity. This new column is coupled to the ProteomeLab PPS Proteome Partitioning System (Beckman-Coulter) for flow through and bound protein collection. For each 250 μl of plasma depleted, 7 ml of flow through protein is collected. In total, 750 μl of plasma from each patient was depleted, providing >1 mg (as 21

25 ml of pooled column flow through) of depleted proteins for downstream RP-HPLC fractionation. Using a 4 ml loop, five separate runs of 4 ml each of the flow-through proteins (collected from depletion of 750 μl of plasma) was fractionated by RP-HPLC (ProteomeLab PPS Proteome Partitioning System) coupled to a C_{18} non-porous column, using a linear acetonitrile gradient, with 60 fractions collected over 30 minutes. The RP-HPLC fractions

30 for each of the five RP-HPLC runs are collected in the same plate to increase the protein concentration of each fraction. Although repeat RP-HPLC fractionation to process the large depleted protein volume (21 ml) is cumbersome, it is superior to the alternative, size exclusion centrifugation. Size exclusion centrifugation results in unacceptable protein loss from (a) non-specific binding of protein to the centrifugation column; and (b) inherent loss of

proteins <10 kDa (which includes vasoactive peptides such as angiotensin II, bradykinin and endothelin, which may be quite important in the pathology of SCI). Protein loss of this magnitude is unacceptable for discovery based approaches as described in this Example. In contrast, repeat RP-HPLC fractionation is extremely reproducible.

5   To determine which peaks of the RP-HPLC chromatogram would be processed for MS, overlay software (32-Karat software, Beckman-Coulter) and newer algorithms (developed in cooperation with Ludesi, Inc.) were used. Both of these tools are particularly useful in later analysis of the discovery cohort. Based on the analysis of 4 SCI and 4 non-SCIRP-HPLC chromatograms, 32 (of 60 total) fractions vary enough between SCI and non-
10   SCI patients for subsequent analysis. *See* FIG. 1.

        Identification and Quantification of Small and Low Abundant Plasma Proteins in SCI. To identify the proteins contained in the RP HPLC fractions described above, LC/MS/MS (LTQ Orbitrap) analysis of each RP HPLC fraction was used to develop a non-SCI/SCI plasma protein database. Briefly, the pooled RP HPLC fractions from a single patient were
15   completely dried using a SpeedVac, resuspended in neutralization buffer and trypsin digested overnight. After trypsin digestion, the RP HPLC fractions were analyzed by LC/MS/MS (Thermo LTQ-Orbitrap). Each RP HPLC fraction contains 50-100 proteins. A database of protein (in Maspectras) was developed and mined for differential protein expression between the two groups and annotated into functional and cell-type specific proteins for subsequent
20   study. Once specific RP-HPLC fractions from ASCI and non-SCI patients were identified to contain potential candidate proteins of interest, proteins in these fractions were quantified using the mass spectroscopy technique, multiple reaction monitoring (MRM). Briefly, after trypsin, samples were submitted for LC/MS/MS identification and quantification using peptide signatures for biomarkers proteins. The Web-based Maspectras database was used to
25   store raw, processed and final proteomic data for analysis and protein profiling.

        Brain Injury Protein Identification. Using the NCBI's list of brain-specific proteins based on EST tags in Unigene (NCBI), 104 brain proteins circulating in children with SCI were identified. *See* Table 2. These peptide identifications have an expectation value of <0.9.

30   Table 2. Brain Injury Protein Biomarkers

Gene Name	Protein Full Name
5HT1A	5-hydroxytryptamine receptor 1A
ACCN4	Isoform 1 of Amiloride-sensitive cation channel 4
AIFM3	Isoform 1 of Apoptosis-inducing factor 3
ANO3	Anoctamin-3

ATP1A3	Sodium/potassium-transporting ATPase subunit alpha-3
B3KXG7	cDNA FU45381 fis, clone BRHIP3021019, highly similar to Homo sapiens protein tyrosine phosphatase,non-re<
BAI3	Brain-specific angiogenesis inhibitor 3
BAIAP3	Isoform 1 of BAI1-associated protein 3
BCAN	Isoform 1 of Brevican core protein
CAMK1G	Isoform 1 of Calcium/calmodulin-dependent protein kinase type 1G
CAMKV	CaM kinase-like vesicle-associated protein
CDH20	Cadherin-20
CHD5	Chromodomain-helicase-DNA-binding protein 5
CHIN	N-chimaerin
CLDND1	Isoform 1 of Claudin domain-containing protein 1
CNDP1	Beta-Ala-His dipeptidase
COKA1	Collagen alpha-1(XX) chain
CTNND2	Isoform 1 of Catenin delta-2
DNAJC18	DnaJ homolog subfamily C member 18
DNM3	Isoform 1 of Dynamin-3
D0CK3	Dedicator of cytokinesis protein 3
DYNC1I1	Isoform 1 of Cytoplasmic dynein 1 intermediate chain 1
ERMN	Ermin
ETBR2	Endothelin B receptor-like protein 2
FAM171A1	Protein FAM171A1
FAM5B	Isoform 1 of Protein FAM5B
FAM5C	Protein FAM5C
FGF1	Isoform 1 of Heparin-binding growth factor 1
FU45872	cDNA FU45872 fis, clone OCBBF3005843
FSTL5	Follistatin-related protein 5
GABBR1	Isoform 1A of Gamma-aminobutyric acid type B receptor subunit 1
GABRA4	Gamma-aminobutyric acid receptor subunit alpha-4
GBRA6	Gamma-aminobutyric acid receptor subunit alpha-6
GFAP	Isoform 1 of Glial fibrillary acidic protein
GNAO	Guanine nucleotide-binding protein G(o) subunit alpha
GPR37	Probable G-protein coupled receptor 37
GPRASP2	G-protein coupled receptor-associated sorting protein 2
GRIN2B	Glutamate [NMDA] receptor subunit epsilon-2
GRM2	Metabotropic glutamate receptor 2
GRM3	Metabotropic glutamate receptor 3
GUCY1B3	Isoform HSGC-1 of Guanylate cyclase soluble subunit beta-1
HEPACAM	Isoform 1 of Hepatocyte cell adhesion molecule
KBTBD11	Kelch repeat and BTB domain-containing protein 11
KCNA1	Potassium voltage-gated channel subfamily A member 1
KCNS1	Potassium voltage-gated channel subfamily S member 1
KCNV1	Potassium voltage-gated channel subfamily V member 1
KIF19	Isoform 1 of Kinesin-like protein KIF19
KIF5A	Kinesin heavy chain isoform 5A
KIF5C	Isoform 1 of Kinesin heavy chain isoform 5C
KLH32	Kelch-like protein 32
KLHL1	Kelch-like protein 1

LGI1	Isoform 1 of Leucine-rich glioma-inactivated protein 1
LRFN2	Leucine-rich repeat and fibronectin type-III domain-containing protein 2
MAGE2	Melanoma-associated antigen E2
MAP2	Isoform 1 of Microtubule-associated protein 2
MAP7D2	Isoform 1 of MAP7 domain-containing protein 2
MLC1	Membrane protein MLC1
MOG	Myelin oligodendrocyte glycoprotein, isoform CRA_d
NAP1L3	Nucleosome assembly protein 1-like 3
NCAM1	Isoform 1 of Neural cell adhesion molecule 1
NDF2	Neurogenic differentiation factor 2
NEUG	Neurogranin
NMDE2	Glutamate [NMDA] receptor subunit epsilon-2
NRG3	Isoform 1 of Pro-neuregulin-3, membrane-bound isoform
NRG3	Pro-neuregulin-3, membrane-bound isoform
NRGN	Neurogranin
OMGP	Oligodendrocyte-myelin glycoprotein
PAQR6	Progestin and adipoQ receptor family member 6
PAX7	Isoform Long of Paired box protein Pax-7
PCDHA9	Isoform 1 of Protocadherin alpha-9
PCDHB10	Protocadherin beta-10
PDE1B	Calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase IB
PHYHIP	Phytanoyl-CoA hydroxylase-interacting protein
PLXNA4	Isoform 1 of Plexin-A4
PNMA3	Isoform 1 of Paraneoplastic antigen Ma3
PRMT8	Protein arginine N-methyltransferase 8
PTN5	Tyrosine-protein phosphatase non-receptor type 5
PTPRN	Receptor-type tyrosine-protein phosphatase-like N
Q59GK5	Glutamate receptor, metabotropic 4 variant
Q75MI7	Putative uncharacterized protein DPP6
RFPL1	Ret finger protein-like 1
RIMS1	Isoform 1 of Regulating synaptic membrane exocytosis protein 1
RP3A	Rabphilin-3A
RTN3	Isoform 1 of Reticulon-3
S39AC	Zinc transporter ZIP12
SC6A7	Sodium-dependent proline transporter
SCN8A	Isoform 1 of Sodium channel protein type 8 subunit alpha
SCN8A	Sodium channel protein type 8 subunit alpha
SERPINI1	Neuroserpin
SH3GL2	Endophilin-A1
SLC1A2	Excitatory amino acid transporter 2
SLC32A1	Vesicular inhibitory amino acid transporter
SLCO1A2	Isoform OATP1a of Solute carrier organic anion transporter family member 1A2
SLIK3	SLIT and NTRK-like protein 3
SNAP91	Isoform 1 of Clathrin coat assembly protein AP180
SNX32	Sorting nexin-32
STXBP1	Isoform 1 of Syntaxin-binding protein 1
SV2B	Synaptic vesicle glycoprotein 2B

TNR	Isoform 1 of Tenascin-R
VA0E2	V-type proton ATPase subunit e 2
WASF1	Wiskott-Aldrich syndrome protein family member 1
WFIKKN2	WAP, kazal, immunoglobulin, kunitz and NTR domain-containing protein 2
WNT16	Isoform Wnt-16b of Protein Wnt-16
WSCD2	Isoform 1 of WSC domain-containing protein 2

Example 3: Biomarker Panel for Diagnosis of Subclinical Brain Injury

Further analysis of the data described herein, as well as data from a cohort of adults having heart surgery, resulted in the following panel of biomarkers that can be used individually, or in combination to diagnose/assess subclinical brain injury.

Table 3. Biomarker Panel for Subclinical Brain Injury

Protein Symbol	Protein Name
ASTN1	Astrotactin1
BAI3	Brain angiogenesis inhibitor 3
CNDP1	Carnosine dipeptidase 1
ERMIN	ERMIN
GFAP	Glial fibrillary acidic protein
GRM3	Glutamate receptor, metabotropic 3
KLH32	Kelch-like protein 32
MAGE2	Melanoma antigen family E, 2
NRG3	Neuregulin 3
NRGN	Neurogranin
OMG	Oligodendrocyte myelin glycoprotein
SLC39A12	Solute carrier family 39 (zinc transporter), member 12
RTN	Reticulon 1
MT3	Metallothionein 3

Example 4: Proteomic Identification of Glial Fibrillary Acidic Protein

as a Plasma Biomarker of Brain Injury in Children with Sickle Cell Disease

Sickle cell disease (SCD) is a chronic hemolytic anemia that is characterized by injury to multiple organs. Stroke is the most prominent injury that can occur to the brain in SCD. Stroke risk has been reduced significantly in children by identifying those patients with elevated transcranial Doppler velocities and implementing chronic red cell transfusion. Adams et al., 339 N. ENG. J. MED. 5-11 (1998). It is currently under study whether risk for brain injury, as ascertained by presence of silent cerebral infarct (SCI) on MRI, can be mitigated by chronic red blood cell transfusion. King et al., 50 PEDIATR. BLOOD CANCER 599-602 (2008). Clearly, biomarkers of stroke risk have transformed care in sickle cell disease.

In children, SCI is an independent risk factor for lower IQ, poorer school performance, and overt stroke. Miller et al., 139 J. PEDIATR. 385-90 (2001); and Bernaudin et al., 15 J. CHILD NERUOL.333-43 (2000). MRI is the only method to identify those patients

33

with SCI. MRI is not an ideal technique to track disease risk because it is expensive, it has limitations on how frequently it can be used, and for some pediatric patients it requires anesthesia, which carries increased risk in SCD, including death. *See* Vicinsky et al., 333 N. ENGL. J. MED. 206-13 (1995). A blood biomarker of SCI would fill a clinical void because

5   blood is easy to obtain and measure, a biomarker may determine risk of or progression of neurologic injury to overt stroke, and a biomarker could benchmark current and new therapies for SCI.

In adults with overt stroke, plasma levels of neuronal and glial proteins are thought to reflect continuous cellular leak from areas of infarction. Biomarkers in this group can be

10 very specific for brain injury. This approach of using brain specific proteins has been taken by investigators to identify biomarkers of overt stroke in adults, with several candidate proteins identified for study. *See* Allard et al., 51 CLIN. CHEM. 2043-51 (2005); Allard et al., 4 PROTEOMICS 2242-51 (2004) However, the identification of SCI from brain-specific proteins leaked into the plasma of SCD patients has not been described.

15 Using a non-biased proteomics approach, glial fibrillary acidic protein (GFAP) was identified in the plasma of SCD patients with SCI who were screened by MRI and proteomic analysis. GFAP is a highly brain-specific intermediate filament protein that is a known biomarker of acute stroke and head trauma in adults. Vos et al., 62 NEUROLOGY 1303-10 (2004); Herrmann et al., 31 STROKE 2670-77 (2000); and Lewis et al., 81 PROC. NATL. ACAD.

20 SCI. U.S.A. 2743-46 (1984). It has been shown to correlate with prognosis and lesion density in these conditions. Nylen et al., 240 J. NEUROL. SCI. 85-91 (2006); Wunderlich et al., 13 EUR. J. NEUROL. 1118-23 (2006). Given the identification of GFAP in the proteomics screen and the available literature describing it, it is therefore hypothesized that GFAP concentrations could serve as a biomarker of brain injury in sickle cell disease.

25   Methods

Patients. A cross-sectional sample of children 5-14 years old with sickle cell disease (HbSS and HbSβ^0) who were screened for the Silent Infarct Transfusion Trial (SIT Trial, ClinicalTrials.gov NCT00072761) were studied (n=259). The SIT Trial is a multi-center, randomized, controlled trial of a three year-transfusion program in children with sickle cell

30 disease and SCI. The primary endpoint includes the occurrence of overt stroke or new or progressive SCI. All patients signed informed consent. SCI is defined by a normal neurologic exam and MRI signal abnormality visible on two views on T2 weighted images. The signal abnormality must measure at least 3 mm in one dimension. SCI status is

adjudicated by a panel of neuroradiologists and neurologists. *See* Vendt et al., 22(3) J. DIGIT. IMAGING 326-43 (2009). Positive and negative control patients were selected from Johns Hopkins Hospital clinics and inpatient units. Positive control plasma samples were obtained from hospitalized children and adults admitted for overt stroke or brain surgery. Negative

5   controls were selected from children 5-16 years old from the Harriet Lane Pediatrics Clinic at Johns Hopkins Hospital. Clinic notes were reviewed to exclude patients with any acute illness, neurologic disorder, or chronic illness other than asthma, obesity, and behavior/mood disorders. De-identified blood samples and clinical data on these controls were obtained through an IRB approved study.

10   Plasma Preparation and Mass Spectroscopic Analysis. Blood was collected into ACD or EDTA tubes and spun at 1500g for 8 minutes per the SIT Trial protocol and stored at -70°C in the Biologic Repository for the SIT Trial at Johns Hopkins University until analysis. Five hundred microliters of plasma was depleted of 12 abundant plasma proteins using an LC10 IgY column (Beckman Coulter, Fullerton, CA) on a ProteomeLab Protein Partitioning

15   System (PPS, Beckman). IgY column flow through was separated into 39 fractions by reverse phase HPLC over a C18 column (Jupiter, Phenomenex) using a continuous acetonitrile gradient (PPS, Beckman). Fractions were dried (SpeedVac, Thermo Scientific, Waltham, MA) and trypsin digested (Promega, Madison, WI) at 37°C overnight. Spectra on each sample were obtained by LC/MS/MS (LTQ-Orbitrap, Thermo Scientific). Spectra were

20   searched for protein identifications using SEQUEST (Sorcerer, SageN, Pennington, NJ) and a human IPI database version 3.4. Post search analysis was performed using Protein Center with a confidence level of < 0.9 as a protein identification cut off.

GFAP Measurements. GFAP was measured in undiluted duplicate plasma samples using an electrochemiluminescent sandwich immunoassay (MesoScale Discovery,

25   Gaithersburg, MD). The monoclonal anti-GFAP blend SMI-26 (Covance, Princeton, NJ) at 100 ng in 30 µL PBS per well was incubated overnight in standard bind MSD plates for capture. Polyclonal anti-GFAP (Dako, Carpinteria, CA) that was directly conjugated with Sulfo-Tag (MesoScale Discovery) was used for detection at 1 µg/ml in PBS. Standard curves were made with 4-fold dilutions of purified GFAP (Calbiochem, La Jolla, CA) in 1% bovine

30   serum albumin (SeraCare Life Sciences, Milford, MA). To minimize analytical variability, control and sickle cell patient samples were assayed on the same plate whenever possible. Assays were analyzed on a Sector Imager 2400 (MesoScale Discovery) according to the manufacturer's protocol.

Statistics. Student's t test was used to compare GFAP concentrations between groups. Natural log-transformed values of GFAP approximated a normal distribution and were used for parametric analyses. Tests of proportion were performed using Fisher's exact test. Analysis was conducted using Stata v10.1 (Stata Corporation, College Station, TX)

5   Results

Mass Spec Identification of GFAP in Plasma. A GFAP peptide was identified by SEQUEST searches in 4 of 6 plasma samples from SIT Trial subjects with SCI. *See* FIG. 2.

Performance and Validation of GFAP Electrochemiluminescent Immunoassay. The GFAP assay was developed using the antibody reagents described by Petzold and adapted to
10   the MSD platform. *See* Petzold et al., 287 J. IMMNOL. METHODS 169-77 (2004). Validation of this method was performed. After experiments to determine the optimal antibody concentrations, plate type, and blocking material, the final assay as used for all the GFAP values reported herein had a lower limit of detection of 0.011 ng/ml as defined by 2 SD above the background of blank wells (n= 19 experiments). The signal to noise ratio was 1.17 at
15   0.01 ng/ml (n= 19 experiments). The lower limit of quantitation, defined as the lowest dilution with a calculated concentration +/- 20% of a known concentration, was 0.026 ng/ml (n= 3 experiments). Inter-assay precision was 2.4% at 10 ng/ml and 3.4% at 0.156 ng/ml (n= 21 experiments). Plasma spiked with GFAP shows 49.8% +/- 22.9% recovery at 10ng/ml, when compared to a standard curve generated in BSA. Whether GFAP was stable in plasma
20   at room temperature was determined for at least 48 hours to confirm that assay values were not an artifact of in vitro proteolysis. Serial measurements from the same aliquot at t= 0, 6, 24, and 48 hours show a CV of 2.5% at 2 ng/ml and 12.1% at 0.2 ng/ml demonstrating the stability of GFAP in plasma.

Plasma GFAP in Sickle Cell Disease and Controls. To validate that the GFAP assay
25   detected elevated plasma GFAP in the context of brain injury, a positive control population composed of plasma samples from children and adults 0-5 days after acute stroke, brain biopsy, or partial brain resection patients was assayed. FIG. 3 shows GFAP levels for stroke (median 0.446 ng/ml, range 0.029-4.29 ng/ml), brain biopsy (median 0.96 ng/ml, range 0.162-1.93 ng/ml), and brain resection (median 1.29 ng/ml, range 0.122-24.1 ng/ml). As the
30   plasma concentration of GFAP in children is unknown, the normal range was established using age-matched controls (5-16 years) from a general pediatric outpatient clinic. Healthy control subjects had blood drawn for screening purposes and were free of acute illness. Of the healthy controls, 46/47 were of African descent. The 95th percentile for GFAP in normal

age matched controls was 0.244 ng/ml. Among positive controls, 74% had GFAP concentrations higher than the 95th percentile of normal controls (p<0.0001).

Comparing the population of SITT participants to healthy controls shows that the two groups have comparable mean concentrations of plasma GFAP (p=0.12). The median GFAP among SITT participants is 0.065 ng/ml (10th percentile 0.026, 90th percentile 0.225), and among healthy controls the median is 0.055 ng/ml (10th percentile 0.025, 90th percentile 0.207); however, several sickle cell subjects have plasma GFAP concentrations much higher than the highest age-matched control value. Using the 95th percentile cutoff of age-matched, healthy controls, 8.9% of the SITT screening samples were elevated (p=0.29). Ten of the SITT screening samples (3.9%) had GFAP levels above the highest healthy, age-matched control level of 0.44 ng/ml (median GFAP of elevated SITT subjects: 1.3 ng/ml, range 0.47 - 4.41 ng/ml) that was comparable to the positive control groups with overt brain injury (brain biopsy, resection or stroke).

Plasma GFAP and SCI. Several studies have evaluated GFAP as a marker of acute brain injury. SCI and stroke are CNS events that are well documented to occur in sickle cell disease. As shown in FIG. 4, children in both the SCI and non-SCI groups had elevated GFAP concentrations. There was no statistical difference in GFAP concentrations between SCI positive and SCI negative patients (p=0.69). The proportion of SCI patients with elevated GFAP above the 95th percentile for age-matched, healthy controls was higher than the proportion of non-SCI patients with GFAP, but this difference is not statistically significant (13.4% vs. 7.6%, p=0.14).

Plasma GFAP in Acute Stroke in SCD. Although the SITT plasma samples are a unique and valuable resource, for validation purposes they pose a particular problem, in that there is no predictable temporal relationship between the plasma sample at entry into the study and development the SCI brain lesion. To further validate GFAP in the childhood SCD population as a marker of subclinical and overt stroke, FIG. 5 shows serial plasma GFAP concentrations from an 11 year-old homozygous sickle cell patient (not a SITT participant) who had an acute stroke during a febrile illness that was clinically suspicious for meningitis. CSF examination was negative for infection and hemorrhage (6 WBC/μL, 2 RBC/μL). The initial GFAP concentration (1.5 ng/ml, 6 times higher than the 95th percentile for normal children) was measured in plasma obtained 32 hours prior to the clinical diagnosis of stroke. The GFAP level in the CSF 25 hours after the clinical diagnosis of stroke was 44.6 ng/ml. For reference, a study of GFAP in the CSF of pediatric all patients <17 years old showed CSF GFAP levels to be 0.1-0.5 ng/ml in the absence of acute injury. Osterlundh et al., 50

PEDIATR. BLOOD CANCER 793-98 (2008). MRI performed 29 hours after the clinical diagnosis of stroke showed right anterior cerebral artery infarct. *See* FIG. 6. This patient also demonstrates that GFAP is likely to be a marker of only acute brain injury, as GFAP levels continued to decrease after red blood cell exchange. One week after the stroke, the patient's sister, an 8 year old also with homozygous sickle cell disease, also developed a febrile illness, during which her plasma GFAP concentration was 0.088 ng/ml.

Plasma GFAP in other sickle cell morbidities. To further explore the specificity of GFAP for brain injury in sickle cell disease, 17 in patients with HbSS had serial plasma samples (n=40) tested for GFAP. Reasons for admission were acute chest syndrome, pain crisis, influenza, fever, aplastic crisis (parvovirus B19 PCR confirmed), and priapism. GFAP was not found to be routinely elevated in sick children with SCD, with concentrations in the normal range for 39/40 samples (range 0.01- 0.24 ng/ml). The highest value (0.44 ng/ml) was observed in a 4 year-old patient admitted for fever. This value is above the 95th percentile of the normal range, but equivalent to the highest normal control value.

<div align="center">Discussion</div>

Using an unbiased screen of the plasma proteome in sickle cell disease, GFAP, a known marker of brain injury, was identified. The discovery of a highly brain specific protein from the plasma in sickle cell patients validates the experimental approach to biomarker discovery used in the present example.

The present example demonstrates that GFAP is a biomarker of cerebral infarct in sickle cell disease, although not a precise biomarker of SCI, as diagnosed by MRI in a cross sectional study of SIT Trial participants, where the timing of SCI is not known. In a cross-sectional population of children 5-14 years old with steady-state sickle cell disease, 3.9% had extreme elevations of GFAP without apparent neurologic injury. It is hypothesized that these children with markedly elevated GFAP levels have subclinical brain injury. By history and neurologic exam, there were no apparent deficits.

SCI is a morbidity defined by either MRI or autopsy. Moser et al., 17 AM. J. NEURORADIOL. 965-72 (1996); and Rothman et al., 20 ANN. NEUROL. 684-90 (1986). SCI in sickle cell disease has been associated with poorer neurocognitive performance than sickle cell disease patients without SCI, but many children with sickle cell disease have poor neurocognitive function, despite normal brain MRIs. Schatz et al., 56 NEUROLOGY 1109-11 (2001); and Armstrong et al., 97 PEDIATRICS 864-70 (1996). Detection of brain injury by plasma levels of proteins highly specific for brain may be a more sensitive means for detecting injury.

SCI is an episodic event resulting from either acute or chronic vascular insult. Plasma GFAP is not elevated in most patients with SCI, and when GFAP is elevated, it is evenly distributed between SCI positive and negative patients. Using cross sectional data, linking an episodic event to a transiently elevated plasma biomarker would be a chance event; therefore,

5   the present inventors conclude that, if GFAP is elevated in the setting of SCI, it is a transient elevation. The sickle cell patient with stroke described herein had an elevated plasma GFAP level for at least 13 days, and perhaps the elevation is shorter in SCI. Other candidate biomarkers may reflect chronic brain and vascular injury that predisposes those with sickle cell disease to SCI and overt stroke.

10   The pre-diagnosis elevation of plasma GFAP in the HbSS patient before diagnosis of overt stroke raises the possibility that GFAP could be used as screening test in ill patients with sickle cell disease. The cerebral infarct was at least a day old at diagnosis, because it could be visualized on non-contrast head CT. Awareness that this child had such a high GFAP level on admission to the hospital might have triggered more rapid evaluation and

15   treatment for evolving brain injury. As plasma GFAP was not elevated in 17 other sickle cell patients admitted for non-neurologic acute problems, a prospective study of the utility of plasma GFAP to aid in the early diagnosis of stroke seems feasible, much as phospholipase A2 is being studied as a predictor of acute chest syndrome. Styles et al., 136 Br. J. Haematol. 343-44 (2007). Approximately 19% of strokes in pediatric sickle cell patients

20   are associated with antecedent medical events (Scothorn et al., 140 J. Pediatr. 348-54 (2002)), and screening on a selected inpatient population may be useful for earlier intervention of evolving stroke.

Plasma is a unique source for biomarker discovery. Advantages are that it contains large quantities of protein, is widely available for study, and it may contain combinations of

25   organ-specific sub-proteomes, with plasma analysis providing information about the state of these tissues. A major challenge to plasma biomarker study in plasma is the high concentration of several proteins that obscure discovery of less abundant candidate proteins. Plasma depletion of abundant proteins has enabled the discovery candidate proteins present in concentrations less than 1 ng/ml.

30   A challenge particular to pediatric proteomic research is the lack of an age-specific reference database. One of the initiatives of the Human Proteome Organisation (HUPO) is the Human Plasma Proteome Project (http://www.hupo.org/research/hppp/). The reference list of 9,504 proteins identified by at least one peptide is the benchmark for all plasma biomarker discovery. However, the application of these data to children is unclear. In the

present example, a repository of age-matched controls was assembled for validation. A future goal is to identify the plasma proteomes of age-matched controls to aid in biomarker discovery for this and all other pediatric proteomic research.

In summary, the results of this study validate the non-biased strategy for identifying potential biomarkers of brain injury in patients with sickle cell disease by identifying GFAP, a known marker of CNS injury in other populations. The identification of GFAP in the plasma of a significant proportion of patients with sickle cell disease with no overt evidence of clinical problems, while not explained by the present study, raises interesting questions about the nature of brain injury in these individuals with SCD. Further proteomic studies of brain-specific proteins in SCD promise to be of great interest to our understanding, detection and treatment of CNS injury in SCD.

Example 5: Plasma Glial Fibrillary Acidic Protein as a Marker of Brain Injury
and Predictor of Neurologic Outcomes after Extracorporeal Membrane Oxygenation

The present example was conducted to determine whether plasma GFAP levels are associated with acute neurologic injury in children undergoing ECMO. This was a prospective observational cohort study of children who underwent ECMO in the Johns Hopkins 26-bed PICU from April 2008 to August 2009. All children age 1 day to <18 years who required ECMO for any indication and who received a continuous unfractionated heparin infusion during the ECMO course were eligible for this study. Twenty two patients were enrolled for GFAP analysis. The median age was 10 days (range 1 day-16 years), 12 (54.5%) patients were males and 12 (54.5%) were African-American. The main illness category was medical non-cardiac 14 (64%) and indications for ECMO were respiratory failure 12 (54.5%), cardiac failure 6 (27.3%), ECPR 3 (13.6%) and sepsis 1 (4.6%).

Median peak GFAP levels (FIG. 7) were higher in children with brain injury than those without (10.2 vs. 0.09ng/ml, p<0.01) and in non-survivors compared to survivors at PICU discharge (5.9 vs. 0.09ng/ml, p=0.01). Using data from all serial samples from each patient, the odds ratio (OR) for brain injury for each 1ng/ml increase in GFAP was 1.5 (95%CI, 1.01-2.16; p=0.046). Abnormal GFAP levels (i.e., >95th percentile) were seen 1-2 days preceding the imaging diagnosis of 2/4 patients with severe neurologic injury or brain death. Plasma GFAP levels remained normal in three patients classified as having an acute neurologic injury based on our a priori definition, as follows: a patient with a right cerebellar hemorrhage diagnosed by head ultrasound who expired on ECMO due to non-neurologic causes, and two patients with small extra-axial hemorrhages: a patient with a small subdural hematoma who survived with good neurologic function and a patient with a grade I

40

intraventricular hemorrhage who also survived with good neurologic function. This initial cohort of ECMO patients included three patients who underwent ECPR: one survived with good neurologic outcome and had normal GFAP levels throughout the ECMO course (median: 0.07ng/ml, IQR: 0.05-0.09ng/ml); one suffered a hypoxic pulseless electrical activity cardiac arrest due to status asthmaticus, evolved to brain death and showed high plasma GFAP levels (median: 27.2ng/ml, IQR: 9.5-44.9ng/ml) and one patient developed severe cerebral edema, eventually had withdrawal of support due to multisystem organ failure and also showed high plasma GFAP levels (median: 5.8ng/ml, IQR: 2.8-10.5ng/ml).

Four out of seven patients with acute neurologic injury had plasma GFAP above the 95th percentile for age-matched controls and 13/15 patients without acute neurologic injury diagnosed during the ECMO course had normal GFAP levels. The two patients without a diagnosis of acute neurologic injury diagnosed during ECMO but with plasma GFAP >95th percentile were infants with normal daily transfontanellar sonograms throughout the ECMO course. However, at 2 weeks and 6 weeks after ECMO decannulation, respectively, one patient was found to have unilateral unilobar focal enchephalomalacia consistent with a prior ischemic event and the other patient had findings of a small, old intraventricular hemorrhage and intraparenchymal hemorrhagic foci. Although exploratory, this analysis yields a receiver operating characteristic (ROC) area in acceptable range for acute neurologic injury on ECMO (0.75, 95%CI: 0.52-0.98).

The results of the present example indicate that GFAP could fill an important clinical gap as a useful diagnostic tool for acute neurologic injury on ECMO and as a predictor of outcome in this high risk group.

<u>Example 6: GFAP as a Marker of Neurologic Injury in Neonates</u>

Hypoxic-ischemic encephalopathy (HIE) is a subset of neonatal stroke occurring in 2.5/1000 term live births. Unfortunately intrapartum fetal monitoring and postpartum brain imaging cannot rapidly identify infants with perinatal brain injury. Importantly, neonates with moderate to severe HIE who receive head cooling within 6 hours of birth may have improved survival with diminished neurodevelopmental disability. Although therapies are being developed to treat the neonate at risk for HIE, no biomarkers are known to acutely identify specific brain injury, follow HIE therapy efficacy or to evaluate new therapies in the child at risk. A rapid test that could be done at the time of birth would be of great clinical benefit in identifying the infant at risk for HIE in order to benefit from these investigational treatments, identify therapeutic efficacy, and provide early prognostic information. Preliminary analysis of 103 prospective admissions to the neonatal intensive care unit over

the past 12 months that were chromosomally normal and without major congenital malformations was conducted. Neonates with neurologic injury which included hypoxi-ischemic encephalopathy (HIE), periventricular leukomalacia, seizures, and cranial bleeding (n=27) to the non-neurologically injured neonates (n=76) were compared. These

5   neurologically injured and non-injured neonates did not differ by gestational age (33.7$\pm$5.7 weeks injured, 33.2$\pm$4.5 weeks non-injured, p=0.67) or birth weight (2264$\pm$1129 grams injured, 1961$\pm$966 grams non-injured, p=0.18). *See* Table 4 below.

Table 4. Patient Characteristics			
	Neurologically Injured	**Non-injured**	**P value**
	N=27	N=76	
Gestational Age (weeks)	33.7$\pm$5.7	33.2$\pm$4.5	0.67
Birth Weight (grams)	2264$\pm$1129	1961$\pm$966	0.18
Cesarean Delivery (%)	59.3	50.0	0.41
Umbilical artery pH	7.12$\pm$0.18	7.23$\pm$0.13	0.007*
Umbilical artery Base Excess (mM)	-9.7$\pm$7.6	-4.7$\pm$4.8	0.001*
Cord pH<7.0 or Base Excess >-12 mM (%)	50	12.1	<0.001*
GFAP on day of life 1 (ng/ml)	0.17$\pm$0.19	0.09$\pm$0.18	0.03*
GFAP on day of life 2	0.12$\pm$0.21	0.15$\pm$0.69	0.86

Neonates with neurologic injury had significantly higher GFAP levels on the first day

10   of life (0.17$\pm$0.19 ng/ml injured, 0.09$\pm$0.18 ng/ml non-injured, p=0.03). *See* Table 4 and FIG. 8. This difference in GFAP levels on the first day of life did not persist on the 2nd day of life (0.12$\pm$0.21 injured, 0.15$\pm$0.69 non-injured, p=0.86) likely reflecting the neurologic injury as finite and not ongoing, which is very useful information to the clinician. Receiver operator characteristic (ROC) curves (FIG. 9) were constructed to determine the optimal cut-

15   off point of GFAP levels within 24 hours of birth to identify neurologic injury. Among neonates with GFAP levels > 0.08 ng/ml the incidence of neurologic injury was 66.7% versus 36.0% among neonates with GFAP levels below this cutoff. A GFAP level > 0.08 ng/ml had a higher area under the ROC curve in identifying neonatal neurologic injury than other commonly used markers for the presence of intrauterine hypoxia-ischemia such as meconium

20   stained fluid, 5 min APGAR <7, or an umbilical arterial gas at the time of birth indicating metabolic acidosis (pH<7.0 and Base Excess < -12 mM). For example, the ROC curve for meconium stained fluid is shown in FIG. 9. Coupled with the sensitive GFAP assay, GFAP can be correlated to functional and structural neurologic injury as a consequence of birth injury. Importantly, the present example validates GFAP as a circulating marker of birth

25   related neurologic injury.

Example 7: Glial Fibrillary Acidic Protein (GFAP) Serves as a Biomarker for Neonatal Neurologic Injury. GFAP is the principal intermediate filament of the human astrocyte and is a marker specific to the central nervous system. Its appearance in a patient's serum has been found to predict neurological outcome in patients after traumatic brain injury and stroke. Brain edema following ischemia causes disruption of the blood-brain barrier, and GFAP is markedly upregulated in astrocytes in the ischemic process. The present objective was to determine if serum levels of GFAP obtained within 24 hours of birth can be used to identify neonates with neurologic injury.

A high sensitivity electrochemiluminescent assay was used to measure GFAP levels in umbilical venous blood at the time of birth and in neonatal blood on day 1 of life for neonates admitted to the neonatal intensive care unit without congenital or chromosomal abnormalities. Receiver operator characteristic (ROC) curves were constructed to determine the optimal cut-off point of GFAP levels on the first day of life to identify a composite variable of neurologic injury that included cranial bleeding and hypoxic-ischemic encephalopathy in order to determine if GFAP levels correlated with neonatal neurologic injury.

Of the 61 neonates that met inclusion criteria, 15 had neurologic injury including 1 case of subarachnoid hemorrhage, 2 cases of subgaleal hemorrhage, 6 cases of intraventricular hemorrhage, and 6 cases of hypoxic-ischemic encephalopathy. Neonates with neurologic injury did not significantly differ in gestational age (33.2 ± 6.2 weeks injured, 33.5 ± 3.4 weeks non-injured), birth weight (2326 ± 1379 grams, 2031 ± 918 grams) or incidence of cesarean delivery (41.2%, 54.5%). GFAP levels at birth in cord blood were 0.16 ± 0.23 ng/ml injured, 0.09 ± 0.11 ng/ml non-injured, $p=0.26$; and on day 1 of life were 0.12 ± 0.06 ng/ml injured, 0.09 ± 0.12 ng/ml non-injured, $p=0.40$. Seventeen patients had elevated GFAP levels above the optimal cutoff point of 0.08 ng/ml which had an area under the ROC curve of 0.81 ($p<0.001$) in the identification of neurologic injury. Neurologic injury was significantly more common in the elevated GFAP group (68.8%) than in those with normal GFAP levels (10.0%, $p=0.02$). *See* FIG. 10.

Further validation of the finding of elevated serum GFAP levels, alone or in aggregate with other clinical markers, at the time of birth or early during the neonatal period, may help identify infants at risk for neurologic injury for appropriate triaging of patients for early therapies and entry into therapeutic trials for these at risk infants.

Example 8: Ability of GFAP to Detect Neurologic Injury in Pediatric Patients with Cardiac Disease on ECMO. Children undergoing cardiopulmonary bypass (CPB) experience

neurologic injury in 30-70% of cases. *See* McQuillen et al., 38 STROKE736-41 (2007); and

Mahle et al., 106 CIRCULATION I-109-I-114 (2002). Extracorporeal membrane oxygenation

(ECMO) represents a similar support modality with a risk of neurologic injury in 10-60% of

cases. *See* Cengiz et al., 33(12) CRIT. CARE MED. 2817-24 (2005); and Ibrahim et al., 69(1)

5   ANN. THORAC. SURG. 186-92 (2000). It is hypothesized that a brain specific protein, glial

fibrillary acidic protein, (GFAP), could serve as a plasma biomarker for neurologic injury in

these vulnerable patients while on ECMO.

          As part of a prospective study of pediatric patients on ECMO, only those patients with

history of critical cardiac disease and ECMO were included in this evaluation. Demographic

10   information such as age, diagnosis, duration of ECMO and outcome at time of PICU

discharge was recorded. Serial blood samples during the ECMO course were evaluated for

GFAP using an electroluminescent assay developed at our institution.

          A total of 7 children with critical cardiac disease were enrolled. Median duration of

ECMO support was 5.2 days (range: 1 to 12 days). The 2 children who underwent ECMO

15   post-CPB had GFAP levels similar to other children who underwent ECMO only. One

patient who experienced acute neurologic injury had plasma GFAP levels that were 100-fold

greater than those without neurologic injury; the increase in plasma GFAP coincided with

detection of neurologic injury by imaging. The plasma GFAP levels during the ECMO

course for each patient are shown in Table 5 below.

Diagnosis		GFAP Measurements						CPB	Neuro	Survival
	pt	1	2	3	4	5	6	XC	Injury	
Heart transplant for DCM s/p bivad, inabilty to separate from CPB	A	0.074	0.43	11.3	20.5			400 min 69 min	Stroke, ICH	No
Dilated Cardiomyopathy	B	0.052	<0.04	0.053					None	Yes
Dilated Cardiomyopathy	C	0.162	<0.04	<0.04					None	Yes
AS,M S	D	<0.04	<0.04		0.06	0.07	0.19		None	Yes
PPHN D-TGA, POD #1 s/p BAS	E	<0.04	<0.04	0.083	0.16				None	Yes
Cardiogenic shock s/p PA stenting, h/o TOF, RV-PA conduit	F	0.068	0.09	0.054					None	Yes
POD #3 RV-PA Conduit for TA, hypoxemia	G	<0.04	<0.04					201 min 130 min	None	Yes

20   Abbreviations: pt-patient, XC-cross clamp, Neuro-neurologic, DCM-dilated cardiomyo-pathy, bivad-
biventricular assist device, min- minutes, ICH-intracranial hemorrhage, AS-aortic stenosis, MS-mitral stenosis,
PPHN-persistent pulmonary hypertension of the newborn, D-TGA-dextro-transposition of the great arteries,
POD-post-operative day, BAS-balloon atrial septostomy, PA-pulmonary artery, TOF-tetralogy of fallot, RV-
25   right ventricle, TA-truncus arteriosus.

Plasma GFAP appears to correlate with neurologic injury in this series of patients with critical heart disease on ECMO. This may aid in detection of neurologic injury in patients on ECMO and during CPB.

Example 9: GFAP is Elevated During Cardiopulmonary Bypass for Repair of

Congenital Heart Disease. Brain injury, manifested as stroke, abnormalities on brain MRI, and neurodevelopmental deficits occur in 30% to 70% of infants after surgical correction of congenital heart defects (CHD). *See* Andropoulos et al., 139 J. THORAC. CARDIOVASC. SURG. 543-56 (2010); and McQuillen et al., 38 STROKE 736-41 (2007). A study of neonatal repairs of one and two ventricle CHD (n=62) reported 39% of patients had injury preoperatively (predominately stroke) and 35% had new lesions post-operatively. *See* McQuillen et al., 38 STROKE 736-41 (2007). The present inventors have examined whether GFAP could serve as diagnostic/prognostic biomarker of brain injury during cardiopulmonary bypass for surgical repair of congenital heart disease. Cardiopulmonary bypass serum samples were obtained before, during (every 30 minutes) and after from 20 consecutive infants (2 days-3 months) undergoing cardiopulmonary bypass for surgical repair of congenital heart disease.

As shown in FIG. 11, the number of cases with quantifiable GFAP levels increase as bypass progresses, beginning in the first hour of bypass (Early Bypass) and peaking at the end of bypass (Late Bypass). Only one case had a quantifiable GFAP level prior to bypass with all 20 cases having measurable values at the end of bypass. As shown in FIG. 12, levels of GFAP are significantly increased in the Mid, Late and Off bypass periods (p<0,05, p<0.001 and p<0.001, respectively when compared to pre- and early bypass levels). The magnitude of elevation in 4 of the cases is equivalent to levels seen with overt stroke (0.5-2.5ng/ml). Two of these 4 infants had a post-operative brain MRI and were confirmed to have an acute stroke. *See also* FIG. 13.

This data provides further evidence for GFAP as a sensitive blood biomarker of brain injury in infants. In addition, it demonstrates that body cooling to 18^0C does not prevent significant GFAP release into the circulation. Like neonates cooled for HIE, GFAP levels peak with re-warming (late bypass period), implying that re-warming is a time of increased vulnerability to injury.

Example 10: GFAP as a Brain Injury Biomarker in Children

Undergoing Extracorporeal Membrane Oxygenation

The objective of this study was to determine whether, in children, plasma glial fibrillary acidic protein is associated with brain injury during extracorporeal membrane oxygenation and with mortality. Prospective patients age 1 day to 18 yrs who required

extracorporeal membrane oxygenation from April 2008 to August 2009 were studied. Glial fibrillary acidic protein was measured using an electrochemiluminescent immunoassay developed at Johns Hopkins. Control samples were collected from 99 healthy children (0.5-16 yrs) and 59 neonatal intensive care unit infants without neurologic injury. In controls, the median glial fibrillary acidic protein concentration was 0.055 ng/ml (interquartile range, 0-0.092 ng/ml) and the 95th percentile of glial fibrillary acidic protein was 0.436 ng/ml. In patients on extracorporeal membrane oxygenation, plasma glial fibrillary acidic protein was measured at 6, 12, and every 24 hrs after cannulation. Twenty-two children who underwent extracorporeal membrane oxygenation were enrolled. Median age was 7 days (interquartile range, 2 days to 9 yrs), and primary extracorporeal membrane oxygenation indication was: cardiac failure, six of 22 (27.3%); respiratory failure, 12 of 22 (54.5%); extracorporeal cardiopulmonary resuscitation, three of 22 (13.6%); and sepsis, one of 22 (4.6%). Seven of 22 (32%) patients developed acute neurologic injury (intracranial hemorrhage, brain death, or cerebral edema diagnosed by imaging). Fifteen of 22 (68%) survived to hospital discharge. In the extracorporeal membrane oxygenation group, peak glial fibrillary acidic protein levels were higher in children with brain injury than those without (median, 5.9 vs. 0.09 ng/ml, $p=.04$) and in non-survivors compared with survivors to discharge (median, 5.9 vs. 0.09 ng/ml, $p=.04$). The odds ratio for brain injury for glial fibrillary acidic protein >0.436 ng/ml vs. normal was 11.5 (95% confidence interval, 1.3-98.3) and the odds ratio for mortality was 13.6 (95% confidence interval, 1.7-108.5).

High glial fibrillary acidic protein during extracorporeal membrane oxygenation is significantly associated with acute brain injury and death. Brain injury biomarkers may aid in outcome prediction and neurologic monitoring of patients on extracorporeal membrane oxygenation to improve outcomes and benchmark new therapies.

<u>Materials and Methods</u>

<u>Study Design</u>. This study was a prospective observational cohort study of children who underwent ECMO in a 26-bed pediatric intensive care unit of a single tertiary care, academic pediatric center from April 2008 to August 2009. Patients <18 yrs who required ECMO for any indication were eligible for this study. This cohort was initiated for the study of coagulation-related risk factors for neurologic injury during ECMO with a secondary aim to investigate brain injury biomarkers in this population. Exclusion criteria were history of heparin-induced thrombocytopenia and use of direct thrombin inhibitors for anticoagulation during ECMO. Parents or legal guardians were approached for consent after patient stabilization, within the first 6 hrs after ECMO cannulation, only when present in the

pediatric intensive care unit. No consent was conducted over the phone. Demographic, clinical, laboratory, imaging, and survival data were collected for each enrolled subject. The ECMO circuit consisted of: custom-packed one-fourth- or three-eighths-inch flexible polyvinylchloride tubing (Medtronic, Minneapolis, MN) with a silicone reservoir, a bladder

5 box (Johns Hopkins Hospital, Baltimore, MD), a 0.8-m^2 to 4.5-m^2 membrane oxygenator (Medtronic), a heat exchanger (Medtronic), and a roller pump (Sorin Cardiovascular USA, Arvada, CO). This study was approved by the Johns Hopkins Institutional Review Board.

Biomarker Sampling and Analysis. Venous blood samples (5 ml in sodium citrate 3.2%) were collected at 6, 12, and 24 hrs after initiation of ECMO and then daily until

10 ECMO discontinuation. After separation by centrifugation within 1 hr, platelet-poor plasma was stored at -80°C. Fifty microliters were used for GFAP measurements in undiluted duplicate plasma samples using an electrochemiluminescent sandwich immunoassay developed on the MesoScale Discovery platform (MesoScale Discovery, Gaithersburg, MD) at Johns Hopkins University and based on the assay of Petzold et al., 287 J. IMMUNOL.

15 METHODS 169-77 (2004). The monoclonal anti-GFAP blend SMI-26 (Covance, Princeton, NJ) was used at 100 ng per well as capture antibody in standard bind plates (MesoScale Discovery) coated either by the manufacturer or in the laboratory with overnight incubation in phosphate- buffered saline. Polyclonal anti-GFAP (Dako, Carpinteria, CA) that was directly conjugated with Sulfo-Tag (MesoScale Discovery) was used for detection at 1 µg/ml

20 in phosphate- buffered saline. Plates were read with a Sector Imager 2400 (MesoScale Discovery). Standard curves were constructed with purified GFAP (Calbiochem, La Jolla, CA) in 1% bovine serum albumin (SeraCare Life Sciences, Milford, MA). After experiments to determine the optimal antibody concentrations, plate type, and blocking material, the final assay for GFAP values had a standard curve with a linear range of quantification from 0.040-

25 40.0 ng/ml. The present GFAP assay had a lower limit of detection of 0.011 ng/ml as defined by two SDs above the background of blank wells (n=19 experiments). Values <0.040 ng/ml were reported as zero. The signal-to-noise ratio was 1.17 at 0.01 ng/ml (n=19 experiments). The lower limit of quantification, defined as the lowest dilution with a calculated concentration ±20% of a known concentration, was 0.040 ng/ml (n=3 experiments).

30 Interassay precision was 2.4% at 10 ng/ml and 3.4% at 0.156 ng/ml (n=21 experiments). Plasma spiked with GFAP shows 49.8% ± 22.9% recovery at 10 ng/ml when compared with a standard curve generated in bovine serum albumin. Validation of the GFAP assay was conducted using discarded diagnostic specimens from normal and positive controls. This

GFAP assay validation study was approved in a separate application by the Johns Hopkins Institutional Review Board with a waiver of consent.

Outcome Measures. The primary independent variable was plasma GFAP elevation above the 95th percentile of normal values in children. The primary outcome was

5   development of acute neurologic injury during ECMO, defined as intracranial hemorrhage, brain infarction, or cerebral edema diagnosed by brain imaging and/or neurologic examination by a pediatric neurologist consistent with brain death while the patient was on ECMO support. It is institutional protocol to obtain daily head ultrasounds for newborns and infants with open fontanelles; older children had brain computed tomography or magnetic

10   resonance imaging studies obtained at the discretion of their physicians. All imaging studies were reviewed by pediatric radiologists as part of routine clinical care. The secondary outcomes were neurologic outcome and survival to discharge from the hospital. Neurologic outcome was measured using the pediatric cerebral performance category (PCPC). *See* Fiser et al., 28 CRIT. CARE MED. 2616-20 (2000); and Fiser et al., 121 J. PEDIATR. 68-74 (1992).

15   The PCPC is a 6-point scale developed from the Glasgow Outcome Scale to assess changes in cognitive abilities in pediatric intensive care. The six PCPC categories are 1) normal, age-appropriate neurodevelopmental functioning; 2) mild cerebral disability, 3) moderate cerebral disability; 4) severe cerebral disability; 5) coma or vegetative state, and 6) brain death. *See* Fiser et al., 28 CRIT. CARE MED. 2616-20 (2000); and Fiser et al., 121 J. PEDIATR. 68-74

20   (1992). A trained pediatric critical care physician assigned PCPC retrospectively by conducting a chart review of the patient's condition at admission to the hospital and at discharge from the hospital. Good neurologic outcome was defined *a priori* as PCPC of 1 or 2 at discharge from the hospital or no change from PCPC at hospital admission.

Statistical Analysis. Exploratory descriptive data analysis was conducted to examine

25   patient and ECMO course characteristics, to describe the distribution of GFAP values among subjects, and to determine the proportion of subjects in whom GFAP levels were above the 95th percentile. The Kruskal-Wallis test was used to compare GFAP concentrations across age categories in normal controls. Patients were divided into two categories for each outcome: those with and without acute neurologic injury, good vs. poor neurologic outcomes

30   at hospital discharge, and survivors vs. non-survivors at hospital discharge. The Mann-Whitney U test was used to compare the median peak GFAP between these groups. Fisher's exact test was used to compare percent of cases above and below 95th percentile of peak GFAP between the groups. Logistic regression with clustering by patient was used to estimate odds of brain injury and death using all serial GFAP data points. For the clustered

analysis, subjects were coded as having no acute neurologic injury until brain injury by imaging or a first neurologic examination consistent with brain death was observed. Subsequent observations were coded as having acute neurologic injury. The odds ratio (OR) and 95% confidence intervals (CIs) are provided. A *p* value of .05 was considered

5   significant. Statistical analysis was conducted using STATA 10.0 (StataCorp, College Station, TX).

Results

Patient Characteristics. Twenty-two of 46 eligible patients were enrolled; 18 patients' parents were not present in the pediatric intensive care unit or it was not possible to conduct a

10   full consent discussion during the 6-hr consent window, five parents declined consent, and one patient had no samples available for testing. Demographic and clinical characteristics of the 22 evaluated patients are presented in FIG. 14.

Individual patient characteristics and outcomes are presented in FIG. 15. Seven of 22 patients (31.8%) had an acute neurologic injury during ECMO based on an *a priori*

15   definition. Using baseline and discharge PCPC, 14 of 22 (63.6%) patients had a good neurologic outcome and eight of 22 (36.4%) patients had a poor neurologic outcome; one had a PCPC >2 and seven died. Causes of death included brain death (one patient), large intracranial hemorrhage (two patients), and withdrawal of mechanical support for medical futility in the face of irreversible multisystem organ failure (four patients).

20   The median duration of ECMO was 12 days (interquartile range [IQR], 5-17 days). The median number of GFAP measurements per patient was 4.5 (range, 1-16). Seventeen patients had three or more measurements, three patients had two measurements, and two patients had only one measurement.

GFAP Results: Controls. In normal pediatric controls with no known neurologic

25   injury, GFAP levels had a median of 0.055 ng/ml (IQR, 0-0.092 ng/ml). GFAP levels were similar across age categories (newborns: median 0.041 ng/ml [IQR, 0-0.096 ng/ml], 6 months to 4 yrs: median 0.046 ng/ml [IQR, 0-0.117 ng/ ml], 5-16 yrs: median 0.057 ng/ml [IQR, 0-0.088 ng/ml], *p*=.7). A 95th percentile cutoff for normal values in children (plasma GFAP ≤0.436 ng/ml) was determined using samples from 158 infants and children: 59 newborns <4

30   days of life in the neonatal intensive care unit without known genetic disorders or intracranial pathology and 99 healthy children 6 months to 16 yrs of age who presented to the Johns Hopkins pediatric outpatient clinic for well-child visits. The assay was further validated for the detection of neurologic injury in patients with brain tumor resection (n=13), brain biopsy

(n=3), and stroke (n=12). GFAP levels in positive controls samples were overall one- to 55-fold higher than for normal controls.

GFAP Results: Patients on ECMO. The median initial GFAP level within 12 hrs after starting ECMO was 0.07 ng/ml (IQR, 0-0.155 ng/ml). There were three of 22 patients with abnormal GFAP concentrations in the first 24 hrs after cannulation; two were patients who sustained cardiac arrest and underwent ECPR with venoarterial cannulation of right neck vessels and one was a patient without known neurologic injury who was placed on venovenous ECMO through a double-lumen right jugular vein cannula. All other 19 of 22 patients had low GFAP levels in the first 24 hrs after cannulation. The median GFAP level on the last ECMO day for those children with three or more samples was 0.07 ng/ml (IQR, 0.053-0.795 ng/ml; n=17). Peak GFAP concentrations were similar comparing newborns with children and infants >30 days (0.155 ng/ml vs. 0.162 ng/ml, respectively, p=.48).

Median peak GFAP levels were significantly higher in children with acute neurologic injury diagnosed during the ECMO course than those without (5.9 vs. 0.09 ng/ml, p=.04) (FIG. 16), in children with poor vs. good neurologic outcome (3.6 ng/ml vs. 0.09 ng/ml, p=.01), and in non-survivors compared with survivors to hospital discharge (5.9 ng/ml vs. 0.09 ng/ml, p=.04). Serial GFAP concentrations in patients with and without acute neurologic injury are displayed in FIG. 17.

Peak plasma GFAP concentrations >95th percentile for normal controls (i.e., >0.436 ng/ml) were noted in six of 22 (27.3%) patients. The proportion of patients with acute neurologic injury was higher in patients with peak GFAP >0.436 ng/ml than in those with peak GFAP ≤0.436 ng/ml (four of six [66.7%] vs. three of 16 [18.8%]; p=.054). Poor neurologic outcome was seen more frequently in patients with peak GFAP >0.436 ng/ml than in those with normal GFAP measurements (five of six [83.3%] vs. three of 16 [18.8%]; p=.01). Similarly, hospital mortality was higher in patients with peak GFAP >0.436 ng/ml than in those with normal GFAP measurements (four of six [66.7%] vs. three of 16 [18.8%], p=.054).

To account for repeated measures per patient, the association of all 126 serial GFAP levels with the outcomes was evaluated using logistic regression clustered by patient. The odds of acute neurologic injury given elevated GFAP (>0.436 ng/ ml) were 11.5 (95% CI, 1.3-98.3). Similar statistically significant results were found for poor neurologic outcome (OR, 25.7; 95% CI, 2.2-298.5) and hospital mortality (OR, 13.6; 95% CI, 1.7-108.5).

After adjusting for neonatal status (≤30 days), the odds of acute neurologic injury remained significantly higher in patients with abnormally elevated plasma GFAP compared

with patients with normal GFAP (adjusted OR, 15.7; 95% CI, 1.8-139.9). In the subgroup of 17 newborns and infants who had daily head ultrasounds performed throughout the duration of ECMO, the unadjusted OR for acute neurologic injury was 22.3 (95% CI, 2.0-245.9).

5   Although exploratory, this analysis yields an area under receiver operating characteristic curve in an acceptable range for acute neurologic injury (area under receiver operating characteristic curve, 0.72; 95% CI, 0.50-0.94), poor neurologic outcome (area under receiver operating characteristic curve, 0.78; 95% CI, 0.58-0.97), and death (area under receiver operating characteristic curve, 0.72; 95% CI, 0.50-0.94).

10   Elevations in GFAP correlated temporally with the imaging diagnosis of brain injury during ECMO. Abnormal GFAP levels (>0.436 ng/ml) were observed 1-2 days before the imaging diagnosis of severe acute neurologic injury or brain death in two of four patients. GFAP levels remained normal in three patients with acute neurologic injury diagnosed by head ultrasound during ECMO, including a patient with a small subdural hematoma and good neurologic function at discharge (PCPC=1), a patient with grade I intraventricular

15   hemorrhage and good neurologic function at discharge (PCPC=1), and a patient with a small right cerebellar hemorrhage who developed multisystem organ failure and ultimately died (FIG. 17). Although it can be speculated that in the first two patients no elevations in GFAP were found because the lesions were minor and extraparenchymal in location, no good explanation exists for a lack in GFAP "response" to a cerebellar intraparenchymal

20   hemorrhage in the third patient. Of note, cause of death in this latter patient was not related to neurologic injury but rather to multisystem organ failure and withdrawal of support resulting from medical futility.

There were two patients without a diagnosis of acute neurologic injury during ECMO but with peak GFAP >95th percentile on the first and the tenth day of ECMO, respectively

25   (FIG. 17). These were newborns with normal daily head ultrasounds throughout the ECMO course. However, at 6 wks and 2 wks after ECMO decannulation, respectively, one patient was found to have small old intraventricular and intraparenchymal hemorrhagic foci and the other had findings of unilateral unilobar focal encephalomalacia consistent with a prior ischemic event on brain magnetic resonance imaging.

30   This initial cohort of ECMO patients included three patients who underwent ECPR; one survived with a good neurologic outcome and had normal GFAP levels throughout the ECMO course (median GFAP, 0.07 ng/ml; IQR, 0.05-0.09 ng/ml); two patients had poor outcomes: one sustained a hypoxic pulseless electrical activity cardiac arrest as a result of status asthmaticus and evolved to brain death (median GFAP, 27.2 ng/ml; IQR, 9.5-44.9

ng/ml) and one patient developed severe cerebral edema, was successfully decannulated from ECMO, but eventually support was withdrawn for multisystem organ failure and medical futility (median GFAP, 5.8 ng/ml; IQR, 2.8-10.5 ng/ml).

<div align="center">Discussion</div>

5   ECMO is a procedure with high risk for brain injury, including intracranial hemorrhage, brain infarction, and brain death. Cengiz et al., 33 CRIT. CARE MED. 2817-24 (2005); Conrad et al., 51 ASAIO J. 4-10 (2004); and Cilley et al., 78 PEDIATRICS 699-704 (1986). The means for timely assessment of such injuries in patients on ECMO are often lacking. Although acute neurologic insult is of great concern in critically ill patients, no brain

10   injury biomarker is available yet for routine clinical practice, although many coordinated efforts are ongoing. *See* Kaneko et al., 80 RESUSCITATION 790-94 (2009); and Laskowitz et al., 40 STROKE 77-85 (2009). The plasma GFAP biomarker used in this study has many advantages such as high specificity to brain, easy to obtain samples for small blood volumes, fast processing, precise quantification, low cost, and minimal technical expertise required for

15   the assay. Serial GFAP measurements could thus be used to monitor neurologic status and response to potential neuroprotective interventions, aid in the prompt diagnosis of acute brain injury, and predict outcomes.

This study demonstrates that plasma concentrations of GFAP are associated with brain injury in children on ECMO. Serial GFAP concentrations appeared stable over time in

20   the absence of neurologic insults and were significantly elevated in patients who were diagnosed with brain injury during ECMO. The majority of patients (19 of 22) had normal GFAP levels in the first 24 hrs after ECMO cannulation, suggesting that cannulation of the right jugular vein±the right carotid artery is not accompanied by injury leading to reactive gliosis and GFAP elevations. GFAP concentrations were elevated in four patients before a

25   diagnosis of brain injury; two patients were diagnosed with acute neurologic injury while on ECMO and two patients had imaging evidence of prior brain ischemia or hemorrhage after ECMO decannulation. This may be particularly important for infants with intraparenchymal lesions that cannot be detected by transfontanellar sonography, thus providing false reassurance to clinicians.

30   Acute neurologic injury is found more frequently in patients undergoing ECPR compared with ECMO for other indications. *See* Barrett et al., 10 PEDIATR. CRIT. CARE MED. 445-51 (2009); and Conrad et al., 51 ASAIO J. 4-10 (2004). Recent studies report 73% survival to hospital discharge in pediatric patients undergoing ECPR with 75-78% of survivors having favorable neurologic outcomes. *See* Barrett et al., 10 PEDIATR. CRIT. CARE

MED. 445-51 (2009); and Prodhan et al., 80 RESUSCITATION 1124-29 (2009). Acute neurologic injury occurs in 22% of pediatric patients undergoing ECPR; of these, 89% die before hospital discharge. Barrett et al., 10 PEDIATR. CRIT. CARE MED. 445-51 (2009). In the present study, normal serial GFAP levels were found in one patient who underwent ECPR

5   and survived with good neurologic outcome. In contrast, two children who underwent ECPR and subsequently developed severe hypoxic brain injury had plasma GFAP levels 20-100 times higher than the 95th percentile for normal children. To the present inventors' knowledge, this is the first report of plasma GFAP as a potential predictive biomarker for ECPR; two prior studies of GFAP after cardiac arrest excluded patients who underwent

10   ECPR. *See* Hayashida et al., 12(2) NEUROCRIT. CARE 252-57 (2010); and Kaneko et al., 80 RESUSCITATION 790-94 (2009). However, these data are very preliminary and no further inferences can be made at this time.

Example 11: GFAP as a Biomarker for Neonatal Hypoxic-Ischemic Encephalopathy Treated with Whole-Body Cooling

15   Subjects. This is an institutional review board-approved prospective cohort study that examined neonates admitted to the neonatal intensive care unit (NICU) at a single tertiary university hospital. Subjects were live-born, non-anomalous, non-syndromic infants born at 36-41 weeks' gestation. This study included neonates born at Johns Hopkins as well as those born within the state of Maryland and transported to the NICU within 6 hours of birth.

20   Records were reviewed to abstract clinical information available at the time of maternal and neonatal discharge. Preeclampsia was defined as proteinuria and new-onset hypertension. Administration of intravenous magnesium sulfate to the mother prior to delivery was recorded because this therapy has been linked with a reduced risk of neonatal brain injury. Rouse et al., 359 N. ENGL. J. MED. 895-905 (2008). Intrauterine growth

25   restriction was defined as an estimated fetal weight less than the 10th percentile for gestational age. Hadlock et al., 181 RADIOLOGY 129-33 (1991). Non-reassuring fetal heart rate tracings were those significant enough to prompt operative vaginal or cesarean delivery. Sepsis was considered present only for neonates with positive blood and/or cerebrospinal fluid cultures.

30   Neonates with moderate to severe encephalopathy that met criteria for whole body cooling were compared with neonates without neurologic injury admitted to the NICU matched by gestational age within 1 week in a 1:1 fashion. Shankaran et al., 353 N. ENGL. J. MED. 1574-84 (2005); and Sarnat et al., 33 ARCH. NERUOL. 696-705 (1976). Neonates of 36 weeks' gestation or longer that qualify are cooled using a conductive water- based

hypothermia system and hypothermia blankets within 6 hours of birth and are kept at a rectal temperature of 33.5°C for 72 hours.

Routinely all neonates with HIE treated with whole-body cooling have a standard neonatal imaging brain MRI prior to discharge from the NICU. For this study, these images were reviewed by an experienced pediatric neuroradiologist (T.A.G.M.H.) blinded to the GFAP results.

Neonates with an abnormal brain MRI were compared with those whose brain MRI was normal. The images were reviewed for focal or diffuse lesions related to hypoxic ischemic injury. MRI brain abnormalities were defined as brain swelling; cortical highlighting; focal or global loss of gray-white matter differentiation; abnormal signal intensity in the basal ganglia and thalami; loss of normal signal intensity in the posterior limb of the internal capsule; acute and subacute parenchymal, intraventricular, or extracerebral hemorrhage; and acutely evolving focal infarction in an arterial territory or in a parasagittal or watershed distribution. *See* Cowan et al., 361 LANCET 736-42 (2003).

Specimens. Specimens collected for measurement of GFAP included umbilical cord blood and neonatal serum. For the umbilical cord blood, a small aliquot was taken from the umbilical cord venous blood sample routinely collected at delivery. For neonatal samples, the remaining fraction of serum from daily laboratory tests was collected after clinically indicated testing was completed. Neonatal serum specimens were collected at the time of admission to the NICU (within 6 hours of birth) and then daily for the first 4 days of life for the non–neurologically injured controls and daily for 7 days for neonates with HIE that underwent whole-body cooling.

GFAP Assay. Using the Mesoscale platform (MesoScale Discovery, Gaithersburg, MD), an electrochemiluminescent sandwich immunoassay was developed for GFAP. *See* Pelinka et al., 57 J. TRAUMA 1006-12 (2004). This was developed after the method of Petzold et al. using a trio of mouse monoclonal antibodies for capture and a rabbit polyclonal for detection. *See* Bembea et al., 11 PEDIATR. CRIT. CARE MED. 723-30 (2010); and Petzold et al., 287 J. IMMUNOL. METHODS 169-77 (2004). Serum samples were assayed in duplicate, and the mean concentration was used for analysis. The lower limit of quantitation was 0.04 ng/ml; values below this were reported as zero.

Statistical Analysis. Comparisons were made using a χ^2 or Fisher's exact test for categorical variables and unpaired Student *t* test for continuous variables. GFAP levels were compared using the Wilcoxon rank-sum test for nonparametric data. Significance was set at

P < .05. Linear regression was used to determine the effect of gestational age on GFAP level in the non-neurologically injured control population.

Receiver-operator characteristic (ROC) curves were constructed to determine the optimal cutoff (as determined by maximal area under the curve) of serum GFAP level at
5   NICU admission to identify HIE qualifying for whole-body cooling and to identify neonates with brain abnormalities on MRI. The area under the ROC curve was used to compare the ability of admission GFAP to identify neonates with abnormalities on brain MRI scans compared with other currently used tests to identify these infants: non-reassuring fetal heart rate tracing, meconium, 5 minute Apgar less than 7, and umbilical arterial pH less than 7.0 or
10   base deficit greater than 12 mM. Statistical analyses were performed with Stata 10 (StataCorp, College Station, TX).

<div align="center">Results</div>

Study Population and Clinical Characteristics. During the period from April 28, 2009, to July 11, 2010, there were 652 admissions to NICU of which 23 consecutive neonates
15   were diagnosed with clinical moderate/severe HIE that qualified for whole-body cooling. These 23 neonates were matched 1:1 by gestational age at birth within 1 week to neonates admitted to the NICU for non-neurological indications. The mean (SD) gestational age for cooled neonates was 38.7 (1.5) weeks and 39 (1.4) weeks for the controls.

Maternal and neonatal characteristics are summarized in FIG. 18. Maternal
20   demographics and incidence of cesarean delivery were not different. The most likely reason for admission for the controls was respiratory distress and to rule out sepsis. The subjects weighed 291 g less at birth, which was statistically, but not clinically, significant and were significantly more likely to have had a placental abruption, metabolic acidosis, a 5 minute Apgar less than 7 and had a longer length of stay in the NICU.

25   GFAP as a Biomarker of HIE. For the controls, a GFAP level at 54 of 138 of the desired time points (39.1%) could not be obtained, and for the cooled subjects 50 of 207 (24.2%) because of an insufficient amount of serum, no blood drawn on that day or an inability to obtain cord blood because the birth occurred at an outside hospital. When neonates with clinical moderate to severe HIE treated with whole-body cooling were
30   compared with controls, mean GFAP levels were significantly elevated in neonatal serum upon admission to the NICU within 6 hours of birth and on days 1, 3, and 4 of life, with *P=.032, P=.013, P=.013,* and *P=.003,* respectively. *See* FIG. 19.

Persistence of serum GFAP was much greater in the HIE group with 6 of 23 controls (26%) and 17 of 23 HIE neonates (74%) having quantifiable GFAP in the serum from day 1

to 4 of life (*P*=.001). GFAP levels on admission to the NICU in the control neonates did not significantly change with gestational age (r=0.22, *P*=.22). Combining all the GFAP values from birth through day 4 of life for the controls showed that the median was 0 ng/ml and the 95th percentile was 0.20 ng/ml. None of the controls had a GFAP value above the 95th

5   percentile vs. 10 of 23 cooled neonates (43.4%) (*P*<.001). Of the 23 cooling subjects, 4 (17.4%) had abrupt elevations of GFAP the day after the 72 hour cooling regimen was completed. *See* FIG. 19.

To test GFAP levels upon NICU admission as a screening test for moderate to severe HIE requiring treatment with whole-body cooling, ROC curves were generated for various

10   thresholds of GFAP level. The NICU admission GFAP of 0.08 ng/ml or greater was the optimal cutoff point to distinguish the groups and produced an area under the ROC curve of 0.709.

Correlation of GFAP with MRI Evidence of Brain Injury and Functional Outcome at Discharge. All 23 neonates that underwent whole body cooling had a clinical brain MRI

15   during their NICU hospitalization. There was no difference in the time after birth that these MRI scans were performed with a mean (SD) of 7.4 (3.7) days of life in the abnormal MRI group and 7.4 (3.9) days in the normal MRI group (*P*=.49). Findings suggestive of HIE were seen in 8 of 23 brain MRI scans (35%). Although those with an abnormal brain MRI were significantly more likely to have been delivered after placental abruption, none of the clinical

20   markers of HIE were associated with an abnormal MRI. *See* FIG. 20.

Serum GFAP levels were consistently elevated in neonates treated with whole body cooling that had abnormal brain MRI scans. *See* FIG. 21. These comparisons were statistically significant on days 1-2 and 4-7 of life, with *P*=.02, *P*=.007, *P*=.001, *P*=.001 and *P*=.007, respectively. In 4 of 8 neonates with an abnormal MRI (50%), a significant increase

25   in GFAP levels the day after cooling ended was observed.

Using ROC analysis, a GFAP of 0.15 ng/ml or greater upon NICU admission was the optimal cutoff to identify neonates with abnormal brain MRI scans among all neonates that underwent whole body cooling (area under ROC curve 0.718). This was superior to indicators currently used to identify intrapartum HIE such as non-reassuring fetal heart rate

30   tracing (area under ROC curve 0.613), meconium (0.508), 5 minute Apgar less than 7 (0.500), and umbilical arterial pH less than 7.0 or base deficit greater than 12 mM (0.500).

To relate GFAP levels to functional outcome, time to oral feeding was used. *See* FIG. 22. For the 15 neonates that underwent whole-body cooling whose MRI was normal, the time to full oral feeds was a mean (SD) of 9.1 (5.4) days. For the 8 neonates with abnormal

MRI scans, 1 died at day of life 7, 5 required a gastric tube at a mean (SD) of 114 (81) days of life, and 2 were on full oral feeds at mean (SD) of 17 (9) days of life. Serum GFAP levels on NICU admission were 0.61 ng/ml for the neonate who died, a mean (SD) of 0.26 (0.10) ng/ml for the neonates discharged with gastric feeding assistance (both of which are above

5   the 95th percentile of GFAP level among non–neurologically injured neonates), and a mean (SD) of 0.04 (0.06) ng/ml for the neonates discharged on full oral feeds (P=.03).

Among all neonates treated with cooling for moderate to severe HIE, serum GFAP level at NICU admission had an area under the ROC curve of 0.731 in predicting an abnormal brain MRI with sensitivity of 50.0%, specificity of 84.6%, positive predictive value

10   of 60.0%, and negative predictive value of 78.6%.

Discussion

The present example describes the first use of a brain-specific protein, GFAP, as a serum biomarker correlating with clinical moderate to severe HIE, development of MRI evidence of brain injury, and functional outcome. Although HIE occurs in 2.5 per 1000 live

15   births at term and 14.5% of all cases of cerebral palsy are associated with intrapartum hypoxia-ischemia, the timing, duration, and outcomes of these injuries are poorly defined. *See* Graham et al., 199 AM. J. OBSTET. GYNECOL. 587-95 (2008).

Biomarkers serve as surrogates of disease injury, evolution, and outcome. Authors of a systematic review of 110 publications in newborns (longer than 36 weeks) that had HIE

20   strictly defined concluded that without biomarkers, the timing, duration, and effectiveness of therapies are ascertained in a relatively blind fashion. *See* Ramaswamy et al., 40 PEDIATR. NEUROL. 215-26 (2009); and American College of Obstetricians and Gynecologists, American Academy of Pediatricians. Chapter 8. Criteria required to define an acute intrapartum hypoxic event as sufficient to cause cerebral palsy. In: Neonatal encephalopathy

25   and cerebral palsy. Washington, DC: ACOG; 2003:73-80. They also concluded that prospective studies should focus on determining which biomarkers best identify neonates who will benefit from intervention.

One study assayed cerebrospinal fluid GFAP levels and found them predictive of neonatal death only, not of abnormal outcomes in survivors; however, blood levels of GFAP

30   were not explored. Blennow et al., 90 ACTA. PAEDIATR. 1171-75 (2001). In the present study, using a new sensitive assay for GFAP, GFAP was found to be an early biomarker of HIE. The control group for this study did not include normal neonates but was representative of the general NICU population of non-premature ill neonates; therefore, the conclusions regarding GFAP as a diagnostic biomarker of HIE may be conservative and highlight the

specificity of GFAP for neonatal brain injury in the context of potentially confounding failure of other organ systems.

Several postnatal biomarkers in blood, cerebrospinal fluid, and urine have been studied to detect HIE, but most have limitations. The urine lactate/creatinine ratio requires specialized nuclear magnetic resonance technology (Oh et al., 153 J. PEDIATR. 375-8 (2008)), cerebrospinal fluid is not amenable to serial sampling (Ramaswamy et al., 40 PEDIATR. NEUROL. 215-26 (2009); and Blennow et al., 90 ACTA. PAEDIATR. 1171-75 (2001)), and other protein biomarkers (e.g., neuron-specific enolase and S100B) have limitations in tissue specificity. *See* Ramaswamy et al., 40 PEDIATR. NEUROL. 215-26 (2009).

Mild hypothermia immediately after hypoxic-ischemic brain injury preserves cerebral energy metabolism, reduces cytotoxic edema, and improves histological and functional outcome. Thoresen et al., 5 SEMIN. NEONATOL. 61-73 (2000). A study of term infants with HIE treated with selective head cooling1 or whole-body cooling (Inder et al., 145 J. PEDIATR. 835-7 (2004)) found that both modes were associated with a decrease in basal ganglia and thalamic lesions, which are predictive of abnormal outcome, and a decrease in severe cortical lesions was seen with selective head cooling. *See* Rutherford et al., 116 PEDIATRICS 1001-6 (2005).

Although cooling in infants with clinical HIE is associated with a reduction in death and neurological impairment (Edwards et al., 340 BMJ c363 (2010)), it is used as a one-size-fits-all therapy because there are no means of monitoring efficacy while it is being delivered. Hypothermia therapy has been shown not to alter serum GFAP levels after severe traumatic brain injury in children. *See* Fraser et al., 12 PEDIATR. CRIT. CARE MED. 319-24 (2011).

As described above, elevated serum GFAP during cooling is significantly associated with evidence of brain injury by MRI. The ability to monitor the level of GFAP during cooling may allow triage of neonates to an escalation of therapy with cooling plus other therapies such as erythropoietin (Kim et al., 23 J. KOREAN MED. SCI. 484-91 (2008)), antiepileptic drugs (Glass et al., 9 CURR. TREAT. OPTIONS NEUROL. 414-23 (2007)), and xenon. *See* Hobbs et al., 39 STROKE 1307-13 (2008). Fifty percent of neonates with brain injury on MRI in this study had a marked increase in levels of GFAP after completion of the 72 hour cooling protocol. These were also the only neonates that had daily increasing levels of GFAP during cooling, making this pattern 100% predictive of an abnormal MRI. This increase after cooling could result from continued brain injury that was suppressed by cooling or could be evidence of rewarming injury.

The dramatic increase in GFAP levels seen at the conclusion of the 72 hour cooling period also raises the question as to what is the optimal duration. At this point, there are no surrogate markers of therapeutic success, and there are no benchmarks to compare new treatments as they are developed. It is possible that circulating brain protein biomarkers such

5   as GFAP will fill this gap.

Although the use of imaging has greatly improved diagnostic accuracy in brain injury, these tests are limited in the early hours after hypoxic-ischemic injury and are difficult to perform in critically ill neonates. Consequently, there are settings in which a rapid blood test for brain injury would be invaluable.

10   MRI is the most common clinical test used for evaluation of neonates with HIE and is associated with outcomes. Barnett et al., 33 NEUROPEDIATRICS 242-8 (2002). The present example demonstrates that levels of GFAP upon NICU admission and during cooling are predictive of an abnormal MRI.

In summary, GFAP may serve as a biomarker to identify and monitor neonates with

15   clinical HIE receiving cooling therapy. Its predictive ability to identify neonates with brain abnormalities because of HIE is better than currently used clinical indicators. Based on the results of this study, GFAP could be used to more specifically and sensitively diagnose brain injury at birth, to facilitate triage of infants into HIE treatment protocols with hypothermia plus adjuvant treatments, serve as an intermediate outcome to benchmark evolving HIE

20   therapies, and give prognostic information to the parents of these at risk children.

Example 12: Proteomic Identification of Thrombospondin-1 and L-Selectin as Plasma
Biomarkers of Subclinical Brain Injury in Children with Sickle Cell Disease

Biomarkers of brain injury have the ability to transform the care of sickle cell disease, as exemplified by transcranial Doppler. It was hypothesized that an unbiased proteomics

25   screen of plasma from sickle cell disease (SCD) patients would yield biomarkers of subclinical brain injury. After depleting plasma of the 12 most abundant proteins, a mass spectrometry-based screen of plasma obtained as part of the Silent Infarct Transfusion (SIT) Trial yielded trombospondin-1 (TSP-1) and soluble L-selectin (SELL). Using a commercial ELISA, TSP-1 and SELL were not different between normal age matched controls (n=23)

30   and SCD patients (n=123). However SCD subjects 5-14 years-old screened for the SIT Trial, a random sampling of SCI (n=62) and non-SCI (n=51) patients demonstrated that TSP-1 and SELL were significantly increased (p=0.013 and p=0.021 respectively). These data validate the experimental approach to biomarker discovery of brain injury in sickle cell disease and describe the first circulating biomarkers of SCI.

Introduction

Sickle cell disease (SCD) is a chronic hemolytic anemia that is characterized by injury to multiple organs. Stroke is the most prominent injury that can occur to the brain in SCD. Stroke risk has been reduced significantly in children by identifying those patients with
5   elevated transcranial Doppler velocities and implementing chronic red cell transfusion. *See* King et al., 50 PEDIATR. BLOOD CANCER 599-602 (2008); and Adams et al., 339 N. ENGL. J. MED. 5-11 (1998). It is currently under study whether risk for brain injury, as ascertained by presence of silent cerebral infarct (SCI) on MRI, can be mitigated by chronic red blood cell transfusion. King et al., 50 PEDIATR. BLOOD CANCER 599-602 (2008). Clearly, biomarkers
10   of stroke risk have transformed care in sickle cell disease.

In children, SCI is an independent risk factor for lower IQ, poorer school performance, and overt stroke. *See* Miller et al., 139 J. PEDIATR. 385-90 (2001); AND MERNAUDIN ET AL., 15 J. CHILD NEUROL. 333-43 (2000). MRI is the only method to identify those patients with SCI. MRI is not an ideal technique to track disease risk because it is
15   expensive, it has limitations on how frequently it can be used, and for some pediatric patients it requires anesthesia, which carries increased risk in SCD, including death. A blood biomarker of SCI would fill a clinical void because blood is easy to obtain and measure, a biomarker may determine risk of or progression of neurologic injury to overt stroke, and a biomarker could benchmark current and new therapies for SCI.

20   Using a non-biased mass spectrometry based proteomics approach, trombospondin-1 (TSP-1) and soluble L-selectin (SELL) were identified in the plasma of SCD patients with SCI who were screened by MRI and proteomic analysis. Both TSP-1 and SELL have been studied in SCD with the roles poorly defined, and in acute brain injury with evidence as important factors for brain recovery (TSP-1) (Lin et al., 34(1) STROKE 177-86 (2003)) or as
25   predictive biomarker (SELL) (Lo et al., 26(9) J. NEUROTRAMA 1479-87 (2009). Given the identification of TSP-1 and SELL in the proteomics screen and the available literature, it is hypothesized that TSP-1 and SELL concentrations could serve as a biomarker of brain injury in sickle cell disease.

Methods

30   <u>Patients</u>. A cross-sectional sample of children 5-14 years old with sickle cell disease (HbSS and HbSβ^0) who were screened for the Silent Infarct Transfusion Trial (SIT Trial, ClinicalTrials.gov NCT00072761) were studied (n=259). The SIT Trial is a multi-center, randomized, controlled trial of a three year-transfusion program in children with sickle cell

disease and SCI. The primary endpoint includes the occurrence of overt stroke or new or progressive SCI. All patients signed informed consent. SCI is defined by a normal neurologic exam and MRI signal abnormality visible on two views on T2 weighted images. The signal abnormality must measure at least 3 mm in one dimension. SCI status is

5 adjudicated by a panel of neuroradiologists and neurologists. Positive and negative control patients were selected from Johns Hopkins Hospital clinics and inpatient units. Positive control plasma samples were obtained from hospitalized children and adults admitted for overt stroke or brain surgery. Negative controls were selected from children 5-16 years old from the Harriet Lane Pediatrics Clinic at Johns Hopkins Hospital. Clinic notes were

10 reviewed to exclude patients with any acute illness, neurologic disorder, or chronic illness other than asthma, obesity, and behavior/mood disorders. De-identified blood samples and clinical data on these controls were obtained through an IRB approved study.

Plasma Preparation and Mass Spectroscopic Analysis. Blood was collected into ACD or EDTA tubes and spun at 1500g for 8 minutes per the SIT Trial protocol and stored at -

15 70°C in the Biologic Repository for the SIT Trial at Johns Hopkins University until analysis. Five hundred microliters of plasma from 15 patients (discovery cohort, n=7 non-SCI and 8 SCI) was depleted of 12 abundant plasma proteins using an LC10 IgY column (Beckman Coulter, Fullerton, CA) on a ProteomeLab Protein Partitioning System (PPS, Beckman). IgY column flow through was separated into 39 fractions by reverse phase HPLC over a C18

20 column (Jupiter, Phenomenex) using a continuous acetonitrile gradient (PPS, Beckman). Fractions were dried (SpeedVac, Thermo Scientific, Waltham, MA) and trypsin digested (Promega, Madison, WI) at 37°C overnight. Spectra on each sample were obtained by LC/MS/MS (LTQ-Orbitrap, Thermo Scientific). Spectra were searched for protein identifications using Xtandem! and a human IPI database version 3.4. Post search analysis

25 was performed using PASS (Integrated Analysis Inc., Bethesda, MD) with a confidence level of < 0.9 as a protein identification cut off.

TSP-1 and SELL Quantitative Measurements. TSP-1 and SELL were measured in diluted duplicate plasma samples using a sandwich ELISA (R&D Systems) according to the manufacturer's protocol. SCI and non-SCI patient samples were picked randomly.

30 Statistics. Student's t test was used to compare plasma concentrations between groups.

<div align="center">Results</div>

Mass Spec Identification of TSP-1and SELL in SCD Plasma. As TSP-1 and SELL function as leukocyte adhesion molecules whose role is poorly understood in SCD

(Ugochukwu et al., 30(4) INT. J. LAB. HEMATOL. 312-16 (2008); and Brittain et al., 97(7) BLOOD 2159-64 (2001)), but circulating SELL has been shown to be a sensitive and specific biomarker of pediatric brain trauma. Using Xtandem! to search the discovery cohort (n=15 patients) spectra, 14 unique peptides for TSP-1 and 3 peptides for SELL in 10 and 13 patients respectively. Xtandem! Log(e) scores ranged from -1.1 to -10.1. Representative spectra for a peptide each for TSP-1 (FIG. 23) and for SELL (FIG. 24) are shown.

Plasma TSP-1 and SCI. Several studies have demonstrated that TSP-1 in situ in the brain is important for recovery of brain injury and that TSP-1 can also play a role increasing the adherence of SCD red cells to endothelial cells. As shown in Table 6 and FIG. 25, TSP-1 levels when compared between normal age matched children and with SCD have no significant difference in concentration (p=0.49). However, as shown in Table 7 and FIG. 26, there was a statistically significant increase in TSP-1 concentrations between SCI positive and SCI negative patients (p=0.013).

Table 6. TSP-1 Levels Control vs. SCD

	SCD, n=123	Control, n=23	P
Mean TSP-1 ng/ml (range)	8928 (724-36500)	6926 (889-26120)	0.49

Table 7. TSP-1 Concentrations in SCI and Non-SCI Children

	SCI, n=62	Non-SCI, n=51	P
Mean TSP-1 ng/ml (range)	10370 (845-36500)	6926 (724-20770)	.013
Mean TSp content ng/million plts (range)	22.95 (3-66)	16.23 (1-56)	.039
Mean plt count	483,000	432,600	.463

Plasma SELL in SCI. SELL is a leukocyte adhesion molecule whose role is poorly understood in SCD, but circulating SELL has been shown to be a sensitive and specific biomarker of pediatric brain trauma. Lo et al., 26(9) J. NEUROTRAUMA 1479-87 (2009). Because SELL was observed in 13/15 of the SITT discovery cohort, SELL was validated as being present in SCD plasma and significance for SCI. SELL mean levels are not significantly different between race and age matched controls and SCD patients. However when SCI and non-SCI groups were compared (FIG. 27), SELL mean levels were significantly elevated in the SCI group (p=0.021).

Discussion

Using an unbiased screen of the plasma proteome in sickle cell disease, two circulating proteins, TSP-1 and SELL, were identified as significantly associated with SCI in children with SCD. The discovery of such specific proteins from the plasma in sickle cell

patients validates the experimental approach to biomarker discovery used in the present example.

The present inventors have demonstrated that TSP-1 and SELL are biomarkers of silent cerebral infarction in sickle cell disease, as diagnosed by MRI in a cross sectional study of SIT Trial participants, where the timing of SCI is not known. In a cross-sectional population of children 5-14 years old with steady-state sickle cell disease, 30% of SCI and 4% of non-SCI had extreme elevations of TSP-1. TSP-1 is a marker known to play a significant role in brain injury repair and angiogenesis. These children with markedly elevated TSP-1 levels have subclinical brain injury by definition as by history and neurologic exam, there were no apparent deficits.

SCI is a morbidity defined by either MRI or autopsy. *See* Moser et al., 17 AM. J. NEURORADIOL. 965-72 (1996); and Rothman et al., 20 ANN. NEUROL. 684-90 (1986). SCI in sickle cell disease has been associated with poorer neurocognitive performance than sickle cell disease patients without SCI (Schatz et al., 56 NEUROLOGY 1109-11 (2001); Armstrong et al., 97 PEDIATRICS 864-70 (1996)), but many children with sickle cell disease have poor neurocognitive function, despite normal brain MRIs. Detection of brain injury by plasma levels of proteins highly specific for brain may be a more sensitive means for detecting injury.

In summary, the results of the present example validate the non-biased strategy for identifying potential biomarkers of brain injury in patients with sickle cell disease by identifying TSP-1, a known marker of CNS injury repair in other populations. The identification of TSP-1 in the plasma of a significant proportion of patients with sickle cell disease and SCI implicate TSP-1 as a biomarker potentially of SCI risk and/or is involved in SCI repair or part of the injury pathway. Further proteomic studies of brain-specific proteins in SCD promise to be of great interest to our understanding, detection and treatment of CNS injury in SCD.

Example 13: Development of Neurogranin Assay

The Identification of Human NRGN Specific Aptamers. Systematic Evolution of Ligands by EXponential enrichment (SELEX) procedure was used to identify the Human NRGN specific aptamers. Briefly, the specific aptamer was selected from a pool of single strand RNA by filter immobilization. The RNA-NRGN target complex can bind to nitrocellulose filter, and free RNA went through filtration. The specific aptamer was recovered from the filter and PCR amplified. After several round of selection, the specific aptamer with highest affinity with NRGN was enriched and sequencing identified.

Prepare the Library for Selection. Single strand DNA oligo pool was chemically synthesized. The DNA oligo has 40mer random central core, which flanked by 2 constant sequences, and the library can be amplified by a pair of primers which target the 5' and 3' conserve ends of the library. The sequence of the library is:

5   5'-TCT CGG ATC CTC AGC GAG TCG TCT G (N40) C CGC ATC GTC CTC CCT A-3' (SEQ ID NO:1)

Generating RNA Library. The single strand DNA library first was annealed with Sel2 5' primer, the sequence is 5'-GGG GGA ATT CTA ATA CGA CTC ACT ATA GGG AGG ACG ATG CGG-3' (SEQ ID NO:2), which contains a T7 promoter for in vitro transcription.

10   The gap was filled in by Klenow reaction, which was performed at 37°C for 1.5 hours. The reaction was purified by phenol: chloroform: isoamyl (25:24:1) and chloroform: isoamyl (24:1) extraction once each and further concentrated with Centricon 30 at 4°C. TE buffer, pH7.4 was used to wash the reaction twice while concentration process. The final OD260 was measured and concentration was determined.

15   The above annealed oligo pool was used to generat RNA library for SELEX by in vitro transcription, using the DuraScribe® T7 Transcription Kits (Epicentre, DS010910), following the manufacture's reaction condition. 2′-Fluorine-CTP (2′-F-dCTP) and 2′-Fluorine-UTP (2′-F-dUTP) were used to replace CTP and UTP in the transcription reaction, the final DuraScript® RNA (2′-fluorine-modified RNA) is completely resistant to RNase A. After in

20   vitro transcription, DNase I was used to treat the reaction and the RNA was extracted with phenol: chloroform: isoamyl (25:24:1) and chloroform once each, followed by concentrate and desalt with Centricon 30 at 4°C.

The RNA was further purified by denatured PAGE (12%, 7M Urea) gel purification. The RNA band was cut from the PAGE gel, RNA was eluted in 2ml TE buffer overnight at

25   4°C. The pure RNA was concentrate again using Centricon 30, and concentration of RNA was determined using conventional method.

Nitrocellulose Filter Pre-clear. A 13mm Swin-Lok Filter holder (Whatman) (13-mm diameter), 0.45um pore size HAWP nitrocellulose disk filters (Millipore) was assembled. The filter was pre-wet with 1 ml washing buffer, which contains 20mM Hepes pH7.4, 50mM

30   NaCl and 2mM CaCl₂. 500pmol RNA was diluted in 100ul 1X binding buffer (the formula is the same as washing buffer except 0.1% BSA was added), and applied into the reservoir of the filter holder. The filter holder was sealed into a 50 ml conical tube and incubated at 37°C for 30 minutes. After incubation, the RNA was recovered by pass through the filter unite

using 1 ml syringe, and 100 µl of 1X binding buffer was used to wash once. The RNA passed through the filter was collected; total pre-cleared RNA was about 180 µl.

Binding Reaction. The binding reaction was assembled by adding 50 pmol human NRGN protein into the pre-cleared RNA, the molecular ratio of RNA:protein was about 10:1. The total volume was brought to 200 µl in 1X binding buffer. The reaction was incubated at 37°C for 15 minutes. A new filter holder was assembled and prewet as above, the binding reaction to the filter was applied, a 5ml syringe was used to push the binding sample through, and the filter was washed with 5ml wash buffer.

Recover the Selected RNA. The filter holder was disassembled, and the filter was transferred into a 1.5ml centrifuge tube which contained 600ul phenol: chloroform: isoamyl (25:24:1). The tube was vortexed vigorously for approximately 1 min, then incubated at RT for 30 minutes. Two hundred microliters of H_2O was added and vortexed again, then spun at 14,000 rpm for 10 minutes. The supernatant, which contained the recovered RNA, was extracted with 400ul chloroform once, then precipitated by adding 500 µl ethanol, 20 µl 3M NaAcetate (pH 5.2) and 3 µl Glycogen blue (Ambion, 5mg/ml), and then incubated at -80°C overnight. The RNA was recovered by centrifugation at 14000 rpm for 20 minutes at 4°C, then washed with 1 ml 75% ethanol once, followed by centrifugation and air drying of the RNA. The dried RNA pellet was dissolved into 20 µl H_2O.

Amplify the Selected RNA by RT-PCR. Five microliters of recovered RNA was used to synthesize the first strand of DNA. Two micromolar of primer Sel2 3' was added into the reaction. The sequence of Sel2 3' primer is: 5'-TCT CGG ATC CTC AGC GAG TCG TC-3' (SEQ ID NO:3). Reverse Transcriptase from Roche (Cat #: 10 109 118 001) was used in the reaction, the conditions were as recommended by the manufacture.

The PCR reaction was assembled as follows: 5 µl first stand DNA (from above step), 3 µl of each 10 µM primers (Sel2 3' from above step and Sel2 5' from above step), 39 µl H2O and 50 µl 2 X TopTaq Master Mix (Qiagen). A total of 8 reactions (800ul) were performed. The PCR cycle condition was as follow: 94°C/5' --> (94°C/30" --> 55°C/30" --> 72°C/30") X 20 cycles --> 4°C. The PCR product was confirmed by 3% agarose gel electrophoresis, and the rest of PCR product were desalted and concentrated using a Centricon 30 at 4°C. The concentration of PCR product was determined by measuring OD/260nm.

Repeat the Selection. One microgram of concentrated PCR product was used to generate RNA for the next round selection, the protocols for in vitro transcription were followed as described above. A total of 10 rounds of selection was performed.

After 10 rounds of selection, another 3 rounds of selection were performed using high-salt binding buffer to increase the selection stringency. The formula for 1X binding buffer F and washing buffer F is the same as the buffers described above, except that the concentration of NaCl was increased to 150mM. The other detailed procedure is the same as that described above.

5

The final PCR product after 13 rounds of selection was cloned into pGEM-T Easy vector (Promega), the enriched aptamers were identified by DNA sequencing. The clones containing the full primers and 40mer insert were aligned using ClustalW2 at EMBL-EBI website.

10   The Identified Human NRGN Specific Aptamers. Six clones were chosen for sequencing and all the sequences' quality were very high. After the DNA alignment analysis, 5 out of the 6 clones were almost identical, except that NRGN-A4 had one nucleotide difference (underlined). The less similar one, NRGN-A6, comparing with the other well aligned 5 clones, showed that all of them have 2 T-rich motifs, which are separated by

15   CC/CA (boxed). NRGN-A1 and NRGN-A6 were selected as targets for validation. The following showed the alignment of the aptamers:

```
NRGN-A1   TCTAACGCCTCCCGTATGTTTT CC T---TTTT-CCATTG---CGGAT  40 (SEQ ID NO:4)
NRGN-A2   TCTAACGCCTCCCGTATGTTTT CC T---TTTT-CCATTG---CGGAT  40 (SEQ ID NO:4)
NRGN-A3   TCTAACGCCTCCCGTATGTTTT CC T---TTTT-CCATTG---CGGAT  40 (SEQ ID NO:4)
NRGN-A5   TCTAACGCCTCCCGTATGTTTT CC T---TTTT-CCATTG---CGGAT  40 (SEQ ID NO:4)
NRGN-A4   TCTAACGCCTCCCG C ATGTTTT CC T---TTTT-CCATTG---CGGAT  40 (SEQ ID NO:5)
NRGN-A6   -TTTTCATTTT C ---A TTTTTTTCCAAATCGATCCGCCGGACCTTAT 43 (SEQ ID NO:6)
```

Validation of the Aptamer-NRGN Interaction. NRGN-A1 and NRGN-A6 RNA were

25   chemically synthesized based on the sequences identified; adding a biotin linker to the RNA 3' end. Two different strategies were used to test NRGN aptamer and NRGN recombinant protein interaction. The details are as described below.

Using Dot Blot to Detect the RNA Protein Complex on Nitrocellulose. Based on the same mechanism that was used in SELEX procedure, RNA-protein complex can be retained

30   on nitrocellulose membrane, a dot blot was used to detect the biotin labeled RNA aptamer. First, a 2-fold series dilution of His-NRGN recombinant protein was made, the amount of protein range from 10 pmol to 0; and then each sample was mixed with 1pmol of NRGN aptamer RNA. The volume of final binding reaction was kept at 20 μl in 1X binding buffer F, the reactions were incubated at 37°C for 15 min.

35   Meanwhile, the dot blot apparatus (Bio-Rad, #170-6545) was setup. The nitrocellulose membrane was cut and shaken in 1X washing buffer F for 30 min prior to use.

The nitrocellulose membrane was put on top of pre-wet Whatman paper and placed on the bottom of the apparatus, then the vacuum was assembled and hooked up. The membrane was washed with 100ul 1X washing buffer F per well once, then the binding reaction was applied. After the reactions were passed through, the membrane was washed with 200ul 1X washing

5   buffer F once, and then drained by vacuuming. The membrane was then UV-crosslinked (Bio-Rad, #165-5031).

The Chemiluminescent Nucleic Acid Detection Module (Pierce, #89880) was used to detect the Biotin labeled RNA aptamer retained on nitrocellulose, following the manufacturer's instruction. Briefly, the membrane was incubated with streptavidin-HRP

10   conjugate, and detected with the chemiluminescent substrate of HRP.

As a result, both NRGN aptamers NRGN-A1 and A6 bound to NRGN protein, the minimum amount of protein needed for detection using this method was 1.25pmol and 0.63pmol respectfully. When the same molar of human albumin was used as control, no signal could be detected, despite the amount of albumin used. This result indicated that both

15   of these 2 aptamers bind to His-NRGN protein specifically. *See* FIG. 28.

Pull-Down Assay. Biotin labeled aptamers were immobilized on streptavidin particles and a pull-down assay was performed. Aptamers were diluted to 1 pmol/µl in TEN100 buffer (10mM Tris-HCl, pH7.5, 1mM EDTA, 100mM NaCl), heated at 65°C for 5', then left at RT for 20 minutes to let the RNA fold into its natural conformation. Streptavidin

20   magnetic particles (Roche, 11641778001) were washed 3 times with twice volume of TEN100 buffer, then the aptamer samples were added and incubated at RT for 30minute with rotation. After incubation, the particles were washed with TEN100 buffer 3 times, and then equilibrated with 1X binding buffer F once. Different amounts of NRGN protein (0-2nmol) were diluted in 1 X binding buffer and added into the aptamer immobilized magnetic

25   particles, then incubated at 37°C for 15 minutes.

After binding incubation, the particles were washed twice with TEN100 buffer. The aptamers protein complex was dissociated by incubating in 26 µl elution buffer (1M NaCl, 10mM EDTA) at room temperature for 10 min. The eluted protein was subjected to SDS-PAGE, and the protein bands were visualized by coomassie staining. The human albumin

30   protein was used as negative control. A typical stained gel is shown in FIG. 29.

Both of the dot blot and pull-down assays showed that the aptamers specifically bind to human NRGN recombinant protein.

Development of a Neurogranin Multiple Reaction Monitoring (MRM) Assay. A neurogranin signature peptide was developed for a mass spectroscopy quantitative MRM

assay. The peptide sequence and transitions are shown in the table below. Labeled GPGPGGPGGAGVAR (SEQ ID NO:7) was spiked in the samples to make standard curve to measure the concentration of signature peptide GPGPGGPGGAGVAR (SEQ ID NO:7). Peptide KGPGPGGPGGAGVAR (SEQ ID NO:8) is also monitored to make sure there is no

5 miscleavage in tryptic digestion.

Table 8. Neurogranin Peptide Sequences

Precursor Q1	Transitions Q3	Peptide ID
553.79	366.18	GPGPGGPGGAGVAR (SEQ ID NO:7)
553.79	423.2	GPGPGGPGGAGVAR (SEQ ID NO:7)
553.79	684.38	GPGPGGPGGAGVAR (SEQ ID NO:7)
553.79	741.4	GPGPGGPGGAGVAR (SEQ ID NO:7)
553.79	798.42	GPGPGGPGGAGVAR (SEQ ID NO:7)
553.79	895.47	GPGPGGPGGAGVAR (SEQ ID NO:7)
553.79	952.49	GPGPGGPGGAGVAR (SEQ ID NO:7)
558.792	366.18	Labeled GPGPGGPGGAGVAR (SEQ ID NO:7)
558.792	423.2	Labeled GPGPGGPGGAGVAR (SEQ ID NO:7)
558.792	694.39	Labeled GPGPGGPGGAGVAR (SEQ ID NO:7)
558.792	751.41	Labeled GPGPGGPGGAGVAR (SEQ ID NO:7)
558.792	808.43	Labeled GPGPGGPGGAGVAR (SEQ ID NO:7)
558.792	905.48	Labeled GPGPGGPGGAGVAR (SEQ ID NO:7)
617.846	684.38	KGPGPGGPGGAGVAR (SEQ ID NO:8)
617.846	741.4	KGPGPGGPGGAGVAR (SEQ ID NO:8)
617.846	798.42	KGPGPGGPGGAGVAR (SEQ ID NO:8)
617.846	950.5	KGPGPGGPGGAGVAR (SEQ ID NO:8)
617.846	962.5	KGPGPGGPGGAGVAR(SEQ ID NO:8)

The signals of neurogranin signature peptide and labeled standard peptide using a ABI Sciex Qtrap 4000 triple quadrapole mass spectrometer are shown in FIG. 30.

10

His-NRGN Recombinant Protein Production. Human NRGN cDNA clone was purchased from Origene (Cat #: RC201209). The coding sequence was cloned into destination vector (Origene, pEX-N-His, Cat # PS100030) by restriction enzymes (Sgf I + Mlu I) fragment swapping to generate pEX-N-His-NRGN expression plasmid. The coding

15 sequence and reading frame were confirmed by DNA sequencing.

pEX-N-His-NRGN plasmid was transformed into Rosetta 2 (DE3) competent cells (Novagen #71397) according to manufacturer's instruction. The bacteria were cultured in the Overnight Express Instant TB Medium (Novagen #71491) at 37°C for 16-18 hours, then harvested and suspended in TEN buffer (50 mM Tris, pH8.0, 0.5 mM EDTA and 0.5 M

20 NaCl). The bacteria were lysated by adding 1% NP-40, 25 mg lysozyme and complete

proteinase inhibitors (Roche), sitting on ice for 30 minutes, then freeze-thaw one time. The
lysate was cleared by centrifugation, then Ni-NTA agarose beads (Qiagen) were added into
the supernatant, and rotated at 4°C for 1 hour. The beads were washed 3 times with washing
buffer (20 mM Imidazole, 20 mM KCl and 0.5 M NaCl). The recombinant protein was

5   eluted off the beads by rotating the beads in the elution buffer (100 mM Imidazole, 20 mM
K_3PO_4 and 167 mM NaCl) at 4°C for 10 minutes. The eluted protein was dialyzed against 3L
PBS overnight, the protein concentration was determined by conventional protein assay.
FIG. 31 shows the typical His-NRGN on PAGE gel after coomassie staining; the predicted
molecular weight of His-NRGN is 8.5 Kd.

10   Development of a Neurogranin Monoclonal. Recombinant neurogranin described
above was used to immunize mice for monoclonal antibody production at Johns Hopkins.
Thirty clones were screened and clone 30.5.2 was identified that bound neurogranin at high
dilution in a direct ELISA shown in FIG. 32.

              Development of a Sandwich ELISA for Neurogranin. A Neurogranin anti-Human

15   monoclonal antibody (Johns Hopkins) at concentration of 75 ng/well was used as a capture
antibody and a rabbit polyclonal to neurogranin (Johns Hopkins) at a concentration of 0.5
µg/ml was used as detection. SULFO-TAG anti rabbit antibody (MSD Cat#R32AB-1) at a
concentration of 1 µg/ml was used as a labeled reporter at a concentration of 1 µg/ml.
GST_NRGN recombinant protein (Johns Hopkins) was used as a standard at a starting

20   concentration of 20 ng/ml then at 1:2 for 7 dilutions in PBS/1%BSA. PBS/1%BSA was used
as blank. The standard curve for this assay is shown in FIG. 33.

              Neurogranin is Biomarker of Acute Brain Injury. Using the neurogranin sandwich
assay described above, serum samples from an infant on ECMO support for 27 days for
cardio-respiratory failure. The infant had normal daily head ultrasounds and at the time of

25   death was thought to not have brain injury. At autopsy, the brain had multiple cortical
infarcts they were not diagnosed by ultrasound. As shown in Figure 34, GFAP levels were
unchanged during the entire coarse of ECMO support. However, neurogranin levels
increased to a peak 15 fold over baseline over 14 days of ECMO support. As neurogranin is
a gray matter, neuronal marker it was more sensitive to cortical gray matter injury than GFAP

30   a marker of white matter injury. This provides evidence that neurogranin is a circulating
biomarker of acute cortical brain injury and in combination with GFAP is able to discrimate
white matter from gray matter injury to the brain.

We claim:

1.   A method for diagnosing subclinical brain injury (SCI) in a patient comprising the steps of:

        a.   collecting a sample from the patient;

5   b.   measuring the levels of one or more biomarkers in the sample collected from the patient; and

        c.   comparing the levels of the one or more biomarkers with predefined levels of the same biomarkers that correlate to a patient having SCI and predefined levels of the same biomarkers that correlate to a patient not having SCI,

10   wherein a correlation to one of the predefined levels provides the diagnosis.

2.   The method of claim 1, wherein the one or more biomarkers is selected from the group consisting of astrotactin 1 (ASTN1), brain angiogenesis inhibitor 3 (BAI3); carnosine dipeptidase 1 (CNDP1); ERMIN; glial fibrillary acidic protein (GFAP); glutamate receptor metabotropic 3 (GRM3); kelch-like protein 32 (KLH32); melanoma antigen family E,2 (MAGE2); neuregulin 3 (NRG3); neurogranin (NRGN); oligodendrocyte myelin glycoprotein (OMG); solute carrier family 39 (zinc transporter), member 12 (SLC39A12); reticulon 1 (RTN1); and metallothionein (MT3).

3.   The method of claim 1, wherein the one or more biomarkers comprises NRGN.

4.   The method of claim 1, wherein the one or more biomarkers comprises OMG.

5.   The method of claim 1, wherein the one or more biomarkers comprises MT3.

6.   The method of any of claims 1-3, wherein the one or more biomarkers further comprises GFAP.

7.   The method of claim 1, wherein the one or more biomarkers comprise NRGN, OMG, and MT3.

8.   The method of claim 5, wherein the one or more biomarkers further comprise GFAP.

9.   The method of claim 2, wherein the one or more biomarkers further comprise the biomarkers listed in Table 1 and Table 2.

10.   The method of claim 1, wherein the sample is a blood, plasma serum, cerebrospinal
5   fluid (CSF), or urine sample.

11.   The method of claim 10, wherein the sample is a blood sample.

12.   The method of claim 10, wherein the sample is a serum sample.
10
13.   A method for diagnosing SCI in a patient comprising the steps of:
        a.   collecting a sample from the patient;
        b.   measuring the levels of a panel of biomarkers in the sample collected from the
patient, wherein the panel of biomarkers comprises ASTN1, BAI3, CNDP1, ERMIN, GFAP,
15   GRM3, KLH32, MAGE2, NRG3, NRGN, OMG, SLC39A12, RTN1, and MT3; and
        c.   comparing the levels of the panel of biomarkers with predefined levels of the
same panel of biomarkers that correlate to a patient having SCI and predefined levels of the
same panel of biomarkers that correlate to a patient not having SCI,
        wherein a correlation to one of the predefined levels provides the diagnosis.
20
14.   A method for diagnosing SCI in a patient comprising the steps of:
        a.   collecting a sample from the patient;
        b.   measuring the levels of a panel of biomarkers in the sample collected from the
patient, wherein the panel of biomarkers comprises NRGN and GFAP; and
25   c.   comparing the levels of the panel of biomarkers with predefined levels of the
same biomarkers that correlate to a patient having SCI and predefined levels of the same
biomarkers that correlate to a patient not having SCI,
        wherein a correlation to one of the predefined levels provides the diagnosis.

30   15.   The method of claim 14, wherein the panel of biomarkers further comprises OMG,
        and MT3.

16.   The method of claim 14, wherein the panel of biomarkers further comprises ASTN1, BAI3, CNDP1, ERMIN, GRM3, KLH32, MAGE2, NRG3, OMG, SLC39A12, RTN1, and MT3.

17.   The method of claims 13 or 14, wherein the sample is a blood, plasma serum, CSF, or urine sample.

18.   The method of claim 17, wherein the sample is a blood sample.

19.   The method of claim 17, wherein the sample is a serum sample.

20.   A method for diagnosing SCI in a patient comprising the steps of:

    a.   collecting a plasma sample from the patient;

    b.   measuring the levels of a panel of biomarkers in the plasma sample collected from the patient, wherein the panel of biomarkers comprises NRGN and GFAP; and

    c.   comparing the levels of the panel of biomarkers with predefined levels of the same biomarkers that correlate to a patient having SCI and predefined levels of the same biomarkers that correlate to a patient not having SCI,

wherein a correlation to one of the predefined levels provides the diagnosis.

21.   The method of claim 20, wherein the panel of biomarkers further comprises OMG, and MT3.

22.   The method of claim 20, wherein the panel of biomarkers further comprises ASTN1, BAI3, CNDP1, ERMIN, GRM3, KLH32, MAGE2, NRG3, OMG, SLC39A12, RTN1, and MT3.

23.   A method for determining the brain injury status in a patient comprising the steps of:

    a.   collecting a sample from the patient;

    b.   measuring the levels of a panel of biomarkers in the sample collected from the patient, wherein the panel of biomarkers comprises ASTN1, BAI3, CNDP1, ERMIN, GFAP, GRM3, KLH32, MAGE2, NRG3, NRGN, OMG, SLC39A12, RTN1, and MT3; and

    c.   comparing the levels of the panel of biomarkers with predefined levels of the same panel of biomarkers that correlate to one or more brain injury statuses selected from the

group consisting of having brain injury, not having brain injury, progressing brain injury, and regressing brain injury,

wherein a correlation to one of the predefined levels determines the brain injury status of the patient.

24.   A method for determining the SCI status in a patient comprising the steps of:

        a.   collecting a sample from the patient;

        b.   measuring the levels of a panel of biomarkers in the sample collected from the patient, wherein the panel of biomarkers comprises ASTN1, BAI3, CNDP1, ERMIN, GFAP, GRM3, KLH32, MAGE2, NRG3, NRGN, OMG, SLC39A12, RTN1, and MT3; and

        c.   comparing the levels of the panel of biomarkers with predefined levels of the same panel of biomarkers that correlate to one or more SCI statuses selected from the group consisting of having SCI, not having SCI, progressing SCI, and regressing SCI,

wherein a correlation to one of the predefined levels determines the SCI status of the patient.

25.   A method for diagnosing brain injury in a patient comprising the steps of:

        a.   collecting a sample from the patient;

        b.   measuring the levels of one or more biomarkers in the sample collected from the patient; and

        c.   comparing the levels of the one or more biomarkers with predefined levels of the same biomarkers that correlate to a patient having brain injury and predefined levels of the same biomarkers that correlate to a patient not having brain injury,

wherein a correlation to one of the predefined levels provides the diagnosis.

26.   The method of claim 1, wherein the one or more biomarkers is selected from the group consisting of astrotactin 1 (ASTN1), brain angiogenesis inhibitor 3 (BAI3); carnosine dipeptidase 1 (CNDP1); ERMIN; glial fibrillary acidic protein (GFAP); glutamate receptor metabotropic 3 (GRM3); kelch-like protein 32 (KLH32); melanoma antigen family E,2 (MAGE2); neuregulin 3 (NRG3); neurogranin (NRGN); oligodendrocyte myelin glycoprotein (OMG); solute carrier family 39 (zinc transporter), member 12 (SLC39A12); reticulon 1 (RTN1); and metallothionein (MT3).

27.   The method of any of claims 1-26, wherein the measuring step comprises immunoassay, immunoblotting method, immunoprecipitation assay, immunostaining method, quantitative assay, immunofluorescent assay, or a chemiluminescence assay.

5   28.   A diagnostic kit for determining brain injury status in a patient comprising:

a.   a substrate for collecting a biological sample from the patient; and

b.   means for measuring the levels of one or more biomarkers selected from the group consisting of ASTN1, BAI3, CNDP1, ERMIN, GFAP, GRM3, KLH32, MAGE2, NRG3, NRGN, OMG, SLC39A12, RTN1, and MT3.

10

FIG. 1

LALDEIATYR, MH+ 1277.7105, m/z 639.3589
080703-ALLENE5.05173.05173.2.dta

SCAN 0; 1.6e+03

CLICK IMAGE TO ZOOM IN; CLICK TOP CORNERS TO ZOOM OUT

b+	#	AA	#	y+	y2+
114.0919	1	L	11		
185.1290	2	A	10	1164.6265	582.8171
298.2131	3	L	9	1093.5893	547.2986
413.2400	4	D	8	980.5053	490.7566
526.3241	5	I	7	865.4783	433.2431
655.3667	6	E	6	752.3943	376.7011
768.4507	7	I	5	623.3517	312.1798
839.4878	8	A	4	510.2676	255.6377
940.5355	9	T	3	439.2305	220.1192
1103.5988	10	Y	2	338.1828	169.5953
	11	R	1	175.1195	88.0637

FIG. 2

FIG. 3

FIG. 4

FIG. 5

FIG. 6

MEDIAN GFAP LEVELS IN PATIENTS ON ECMO (n≈22)

$p \leq 0.01$

$p \leq 0.01$

GFAP (MEDIAN, ng/ml)

INJURY   NO INJURY   DIED   SURVIVED

FIG. 7

$p = 0.03$

GFAP (ng/ml)

NON-INJURED   INJURED

FIG. 8

FIG. 9

AREA=0.79

AREA UNDER ROC CURVE= 0.7860
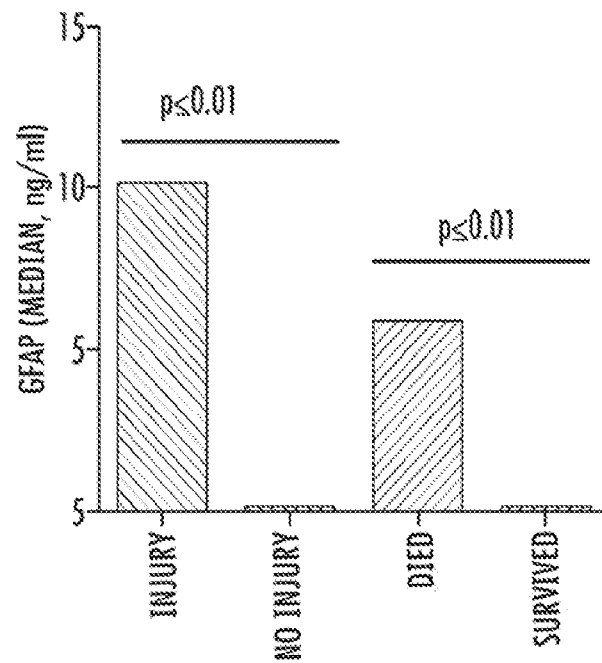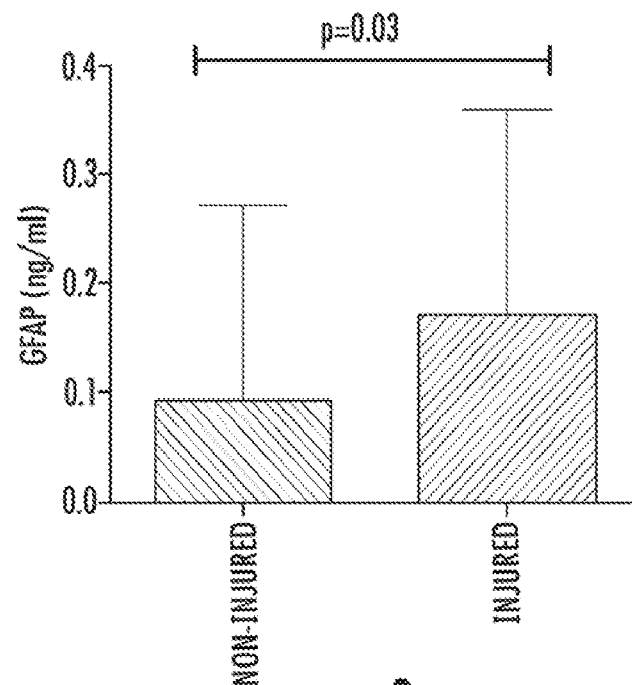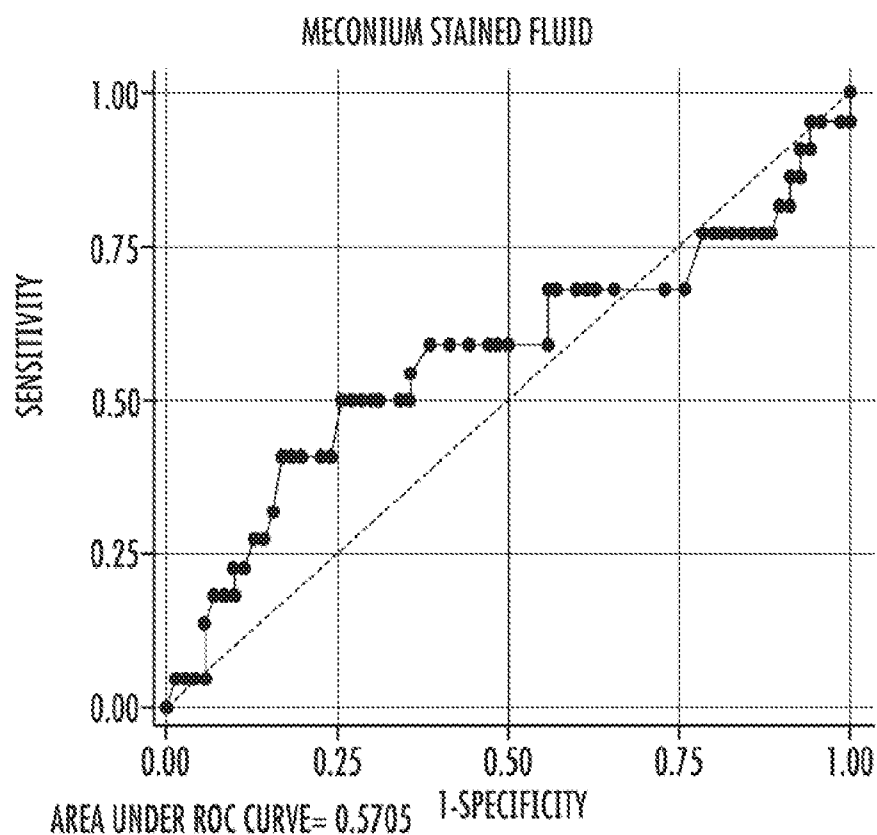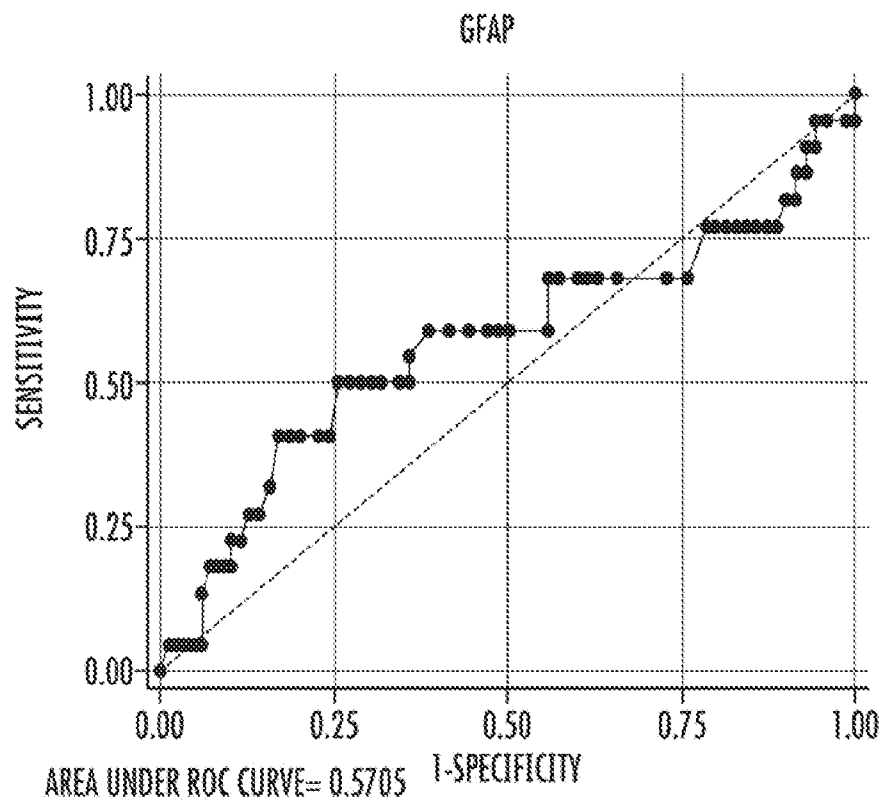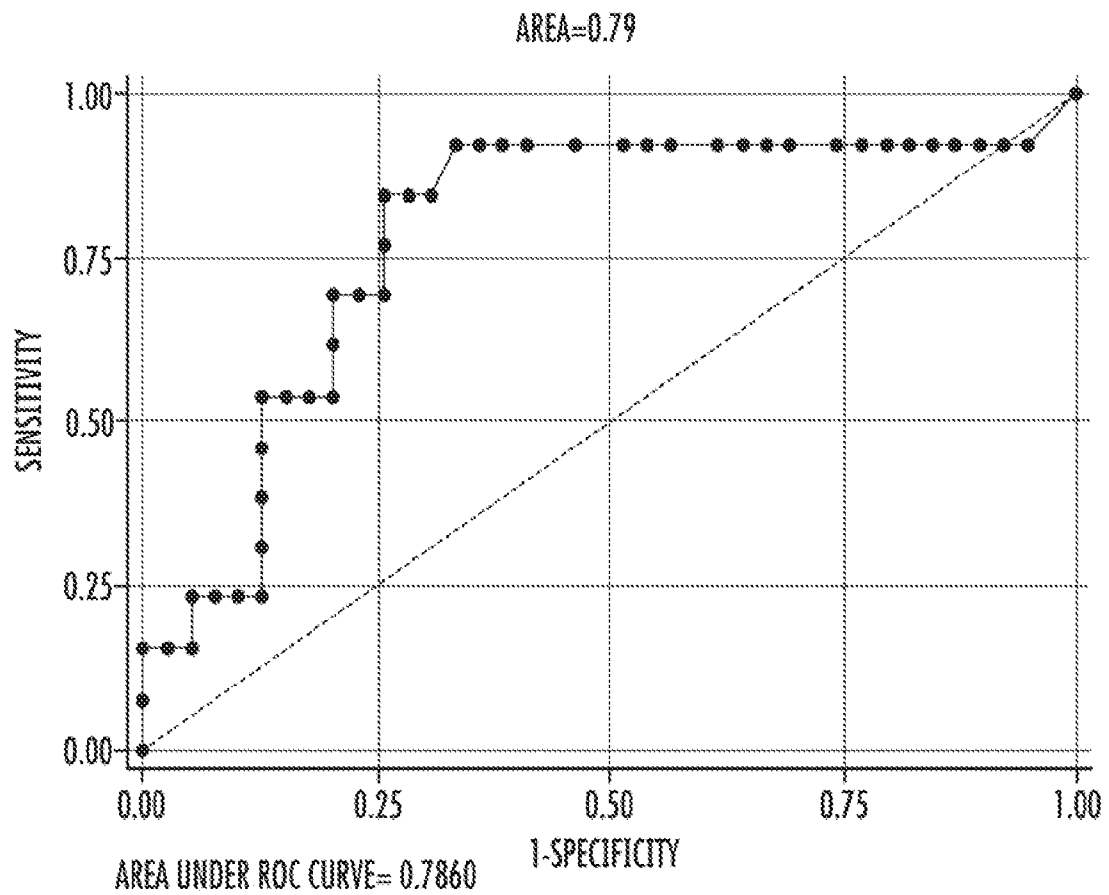
SENSITIVITY	Pr(+ D)	15.38%
SPECIFICITY	Pr(- D)	94.87%
POSITIVE PREDICTIVE VALUE	Pr(D +)	50.00%
NEGATIVE PREDICTIVE VALUE	Pr(-D -)	77.08%

FIG. 10

FIG. 11

GFAP DURING CHD SURGERY

FIG. 12

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FIG. 13
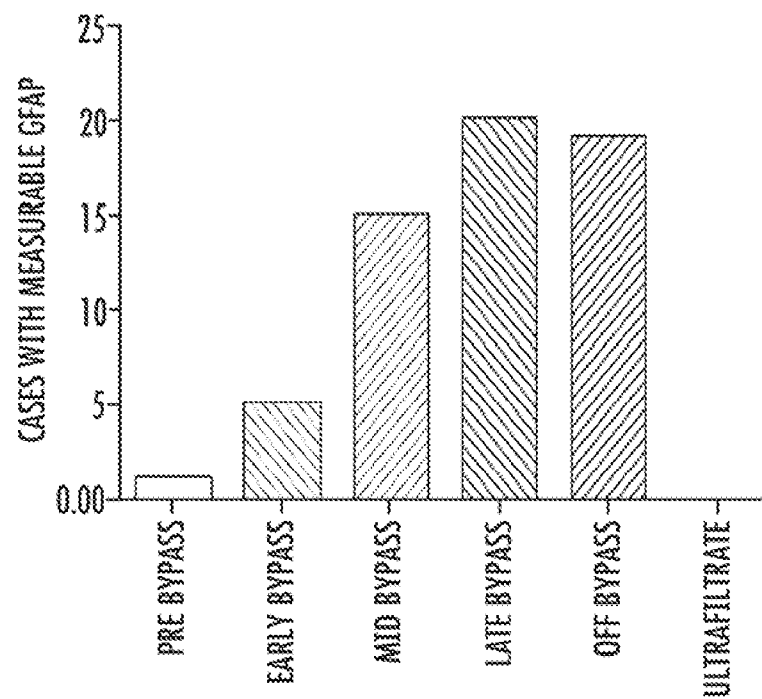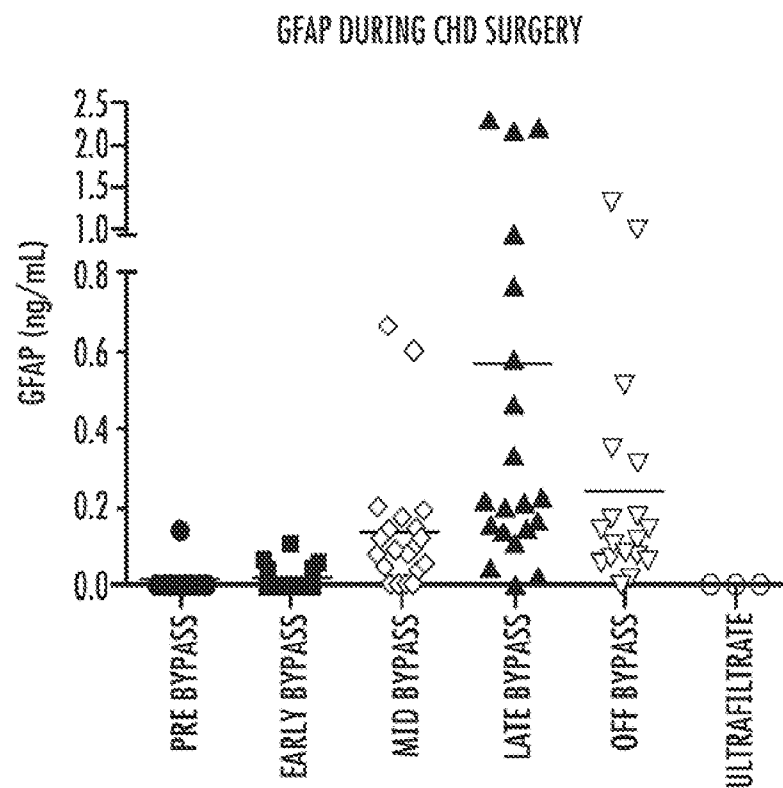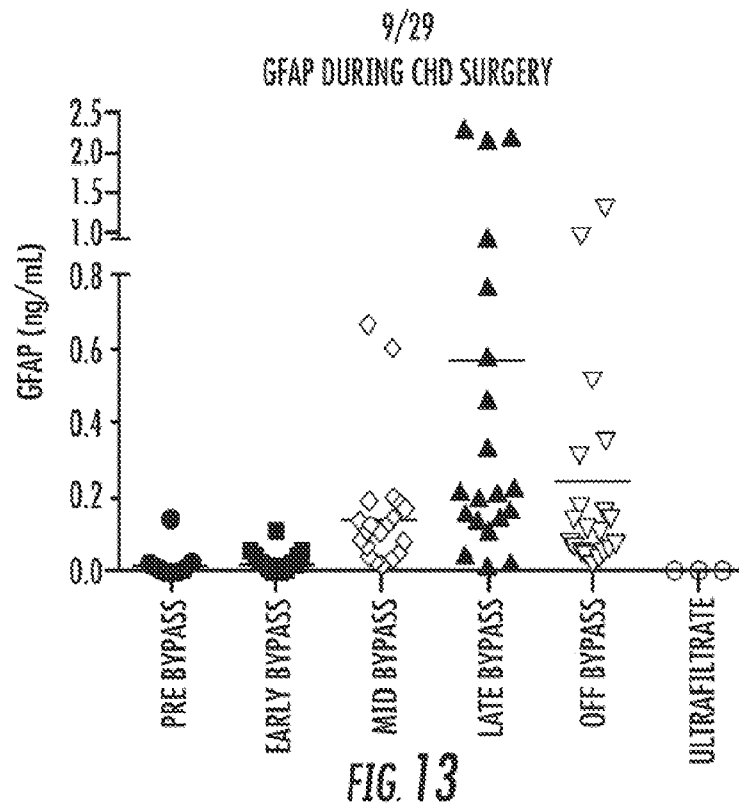
PATIENT CHARACTERISTICS		ECMO COURSE CHARACTERISTICS	
Age, median (range)	10 days (2 days to 16 yrs)	Primary indication for ECMO, NO. (%)	
Male, no. (%)	12 (54.5)	Cardiac failure	6 (27.3)
Race, no. (%)		Respiratory failure	12 (54.5)
Black	12 (54.5)	Cardiac arrest/ECPR	3 (13.6)
White	7 (31.8)	Sepsis	1 (4.6)
Other	3 (13.6)	Mode of extracorporeal support, no. (%)[b]	
Hispanic, no. (%)	2 (9.1)	VA ECMO	17 (77.3)
Weight, kg, median (IQR)	3.8 (3.1, 6.9)	VV ECMO	3 (13.6)
Illness category, no (%)		VV to VA ECMO	2 (9.1)
Medical cardiac	5 (22.7)	Complications on ECMO, NO. (%)	
Medical noncardiac	14 (63.6)	Acute neurologic injury	7 (31.8)
Surgical cardiac	1 (4.6)	Intracranial hemorrhage	5 (22.7)
Surgical noncardiac	2 (9.1)	Severe cerebral edema	1 (4.6)
PICU length of stay, median (IQR)	15 days (8-23 days)	Brain death	1 (4.6)
Hospital length of stay, median (IQR)	30 days (19-53 days)	Pulmonary hemorrhage	2 (9.1)
Mortality before PICU discharge, no. (%)	7 (31.8)	Renal replacement therapy, no. (%)	14 (637.6)
ECMO to ANI diagnosis, median (IQR)	82 hrs (69-104 hrs)	Duration of ECMO support, median (IQR)	114 hrs (86 hrs to 12 days)

ECMO, extracorporeal membrane oxygenation; IQR, interquartile range; PICU, pediatric intensive care unit; ANI, acute neurologic injury; ECPR, extracorporeal cardiopulmonary resuscitation; VA, venoarterial; VV, venovenous

[b] All ECMO cannulations were performed through the right jugular vein and right carotid artery for VA ECMO and through the right jugular vein using a double-lumen cannula for VV ECMO. None of the patients enrolled in this study underwent direct intracardiac cannulation.

FIG. 14

Patient No.	Primary Diagnosis	ECMO Indication	ECMO Duration	No. of GFAP Measurements	Peak GFAP	ANI Diagnosed During ECMO
1	Liver failure, CPA posttransplant	Cardiac Disease	4 days	5	5.86	Unilateral IVH, intraparenchymal (basal ganglia, right temporal and occipital) hemorrhage, 2-cm midline shift, edema
2	Dilated cardiomyopathy	Cardiac Disease	3 days	4	20.5	Frontoparietal 4 x 5 x 4-cm intraparenchymal hemorrhage, 2.5-cm midline shift, unilateral SDH, SAH, IVH
3	Dilated cardiomyopathy	Cardiac Disease	5 days	3	0.053	No
4	PPHN	Respiratory Disease	6 days	4	0.164	No
5	Septic shock	Sepsis	1 day	1	0ª	No
6	PPHN	Respiratory Disease	5 days	8	0.088	No
7	PPHN	Respiratory Disease	4 days	5	0.081	No
8	Dilated Cardiomyopathy	Cardiac Disease	1 day	2	0.162	No
9	PPHN	Respiratory Disease	7 days	7	0.062	No
10	Critical aortic stenosis	Cardiac Disease	12 days	6	0.194	No
11	PPHN	Respiratory Disease	30 days	16	1.43	No
12	Diaphragmatic eventration	Respiratory Disease	14 days	11	0.398	Unilateral cerebellar hemisphere, 1.5 x 2-cm intraparenchymal hemorrhage
13	TOF, Pulmonary atresia	ECPR	4 days	3	0.090	No
14	Status asthmaticus	ECPR	4 days	2	44.9	Rapid progression to brain death
15	PPHN	Respiratory Disease	12 days	3	0.795	No
16	PPHN	Respiratory Disease	15 days	11	0.111	Small SDH along the falx cerebri
17	PPHN	Respiratory Disease	4 days	3	0.155	No
18	CDH	Respiratory Disease	18 days	13	0.400	No
19	Truncus arteriosus	Cardiac Disease	4 days	7	0ª	No
20	PPHN	Respiratory Disease	3 days	7	0ª	Unilateral subependymal grade I IVH
21	CDH	Respiratory Disease	17 days	8	0ª	No
22	Neonatal enterovirus sepsis	ECPR	8 days	8	14.6	Severe diffuse cerebral edema

ECMO, extracorporeal membrane oxygenation; GFAP, glial fibrillary acidic protein; ANI, acute neurologic injury; PCPC, Pediatric Cerebral Performance Category; PICU, pediatric intensive care unit; CPA, cardiopulmonary arrest; PPHN, persistent pulmonary hypertension of the newborn; TOF, tetralogy of Fallot; CDH, congenital diaphragmatic hernia; ECPR, extracorporeal cardiopulmonary resuscitation; IVH, intraventricular hemorrhage; SDH, subdural hemorrhage; SAH, subarachnoid hemorrhage; HCT, head computed tomography; HUS, head ultrasound; BD, brain death.

ªValues below the lower limit of quantification (LLQ=0.040 ng/mL); ᵇnormal serial HUS during ECMO. Brain magnetic resonance imaging 6 weeks post-ECMO decannulation: few old intraventricular and intraparenchymal hemorrhagic foci; ᶜnormal serial HUS during ECMO. Brain magnetic resonance imaging 2 weeks post-ECMO decannulation: unilateral unilobar focal encephalomalacia consistent with prior ischemic event.

FIG. 15

ANI Diagnostic Modality	ECMO to ANI Diagnosis	ECMO to GFAP >95th Percentile	Baseline PCPC	Discharge PCPC	Neurologic Outcome	PICU Survival
HCT	104 hrs	24 hrs	1	6	Poor	No
HUS	11 hrs	19 hrs	2	6	Poor	No
----	----	----	1	1	Good	Yes
---	---	---	1	2	Good	Yes
---	---	---	1	6	Poor	No
---	---	---	2	2	Good	Yes
----	---	---	1	1	Good	Yes
----	----	----	1	1	Good	Yes
----	----	---	1	2	Good	Yes
----	---	---	1	1	Good	Yes
---	---	12 hrs[b]	1	3	Poor	Yes
HUS	82 hrs	---	1	6	Poor	No
---	---	---	1	2	Good	Yes
8D examination	64 hrs	12 hrs	1	6	Poor	No
----	---	10 days[c]	1	2	Good	Yes
HUS	10 days	---	1	1	Good	Yes
----	---	----	1	1	Good	Yes
---	----	---	1	6	Poor	No
---	----	----	1	1	Good	Yes
HUS	69 hrs	---	1	1	Good	Yes
---	---	----	1	1	Good	Yes
HUS	2 hrs	6 hrs	3	6	Poor	No

FIG. 15 (cont.)
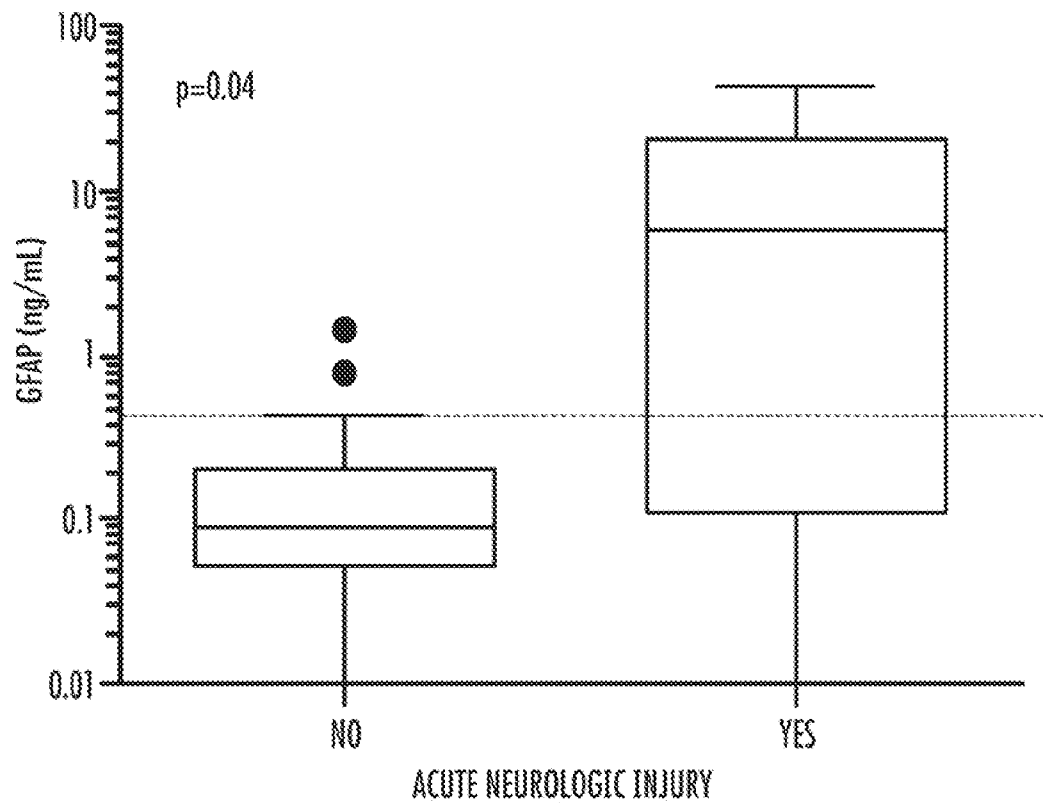
PEAK PLASMA GLIAL FIBRILLARY ACIDIC PROTEIN
(GFAP) CONCENTRATIONS IN CHILDREN ON EXTRACORPOREAL
MEMBRANE OXYGENATION WITH AND WITHOUT ACUTE
NEUROLOGIC INJURY (n=22). PLEASE NOTE LOGARITHMIC
SCALE ON Y-AXIS.

FIG. 16

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ACUTE NEUROLOGIC INJURY

FIG. 17A

NO ACUTE NEUROLOGIC INJURY

FIG. 17B
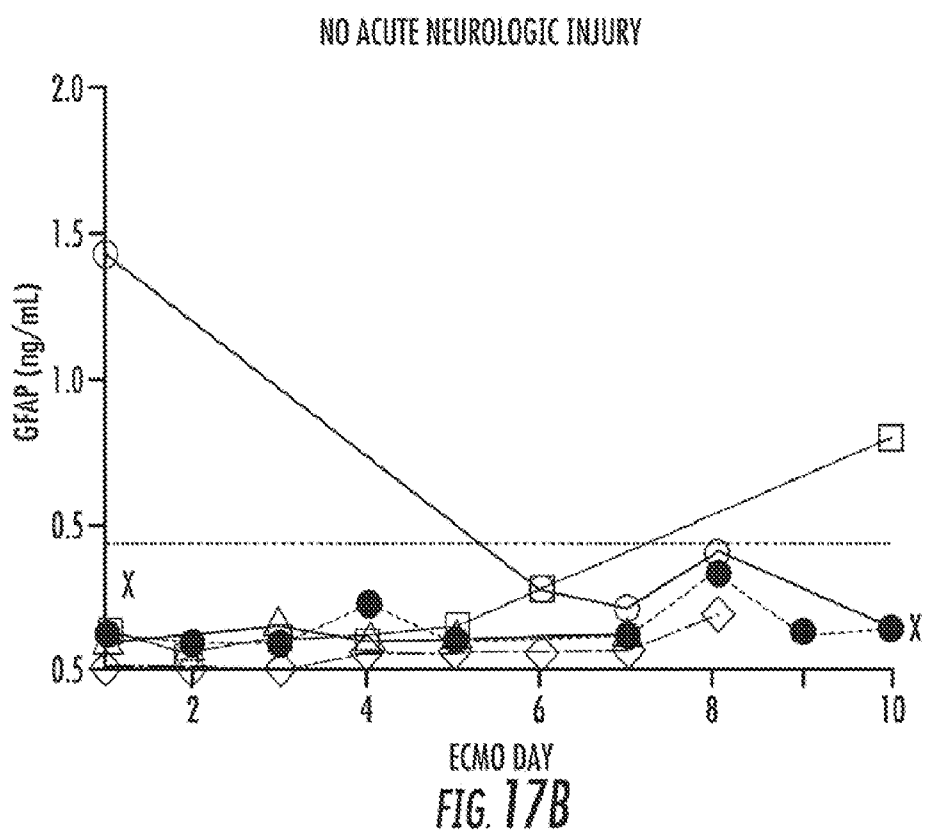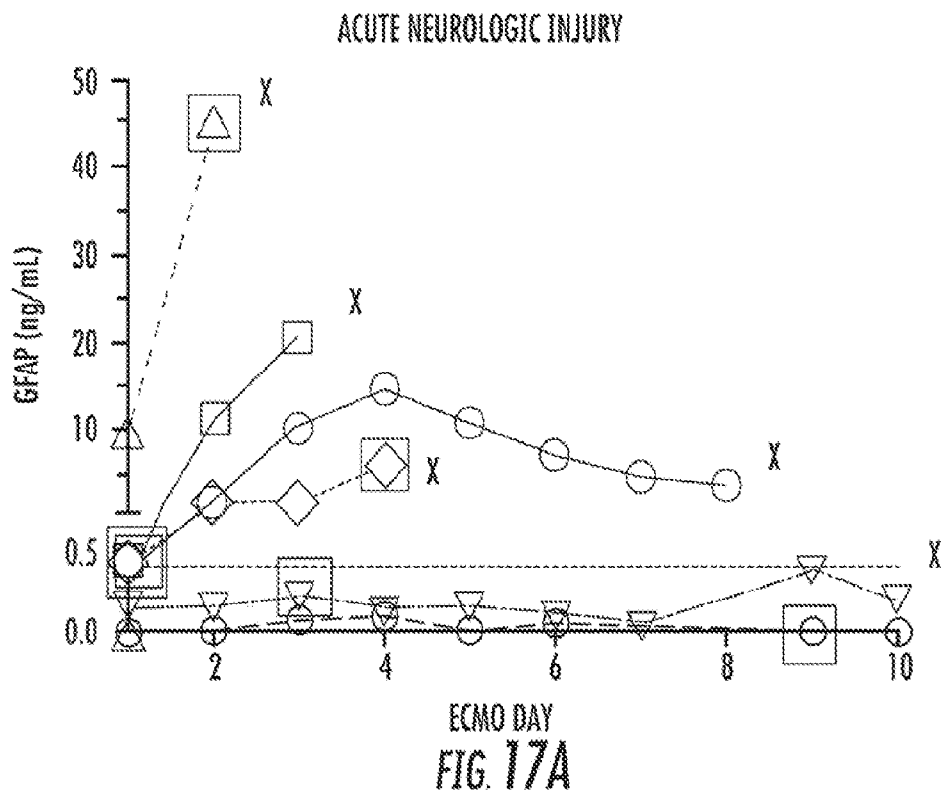
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TABLE 1 MATERNAL AND NEONATAL CHARACTERISTICS			
CHARACTERISTIC	COOLING (n=23)	COOLINGS (n=23)	P VALUE
MATERNAL AGE, y	26.2±7.1	26.9±6.9	.739
MATERNAL RACE			.244
WHITE	9 (39.1%)	5 (21.7%)	
BLACK	11 (47.8%)	17 (73.9%)	
OTHER	3 (13%)	1 (4.3%)	
NULLIPAROUS	14 (60.9%)	8 (34.8%)	.078
CESAREAN DELIVERY	15 (65.2%)	12 (52.2%)	.369
TWIN GESTATION	1 (4.3%)	0	1.00
PREECLAMPSIA	3 (13%)	3 (13%)	1.00
MAGNESIUM EXPOSURE	1 (4.3%)	3 (13%)	.608
INTRAUTERINE GROWTH RESTRICTION	4 (17.4%)	2 (8.7%)	.665
CLINICAL CHORIOAMNIONITIS	3 (13%)	4 (17.4%)	1.00
MECONIUM STAINING (MODERATE-THICK)	6 (26.1%)	7 (30.4%)	.743
NONREASSURING FHR TRACING	11 (47.8%)	6 (21.6%)	.127
ABRUPTION	8 (34.8%)	1 (4.3%)	.022[a]
BIRTHWEIGHT, g	2978±630	3269±655	.132
UMBILICAL ARTERY pH	6.92±0.19	7.14±0.13	< .001[a]
UMBILICAL ARTERY BASE DEFICIT, mM	15.5±6.7	7.8±5.9	.001[a]
CORD pH <7.0 OR BASE DEFICIT >12	16/19 (84.2%)	3/17 (17.6%)	< .001[a]
5 MINUTE APGAR <7	21 (91.3%)	6 (21.6%)	< .001[a]
RESPIRATORY DISTRESS	11 (47.8%)	16 (69.6%)	.134
SEPSIS (PLUS BLOOD OR CSF CULTURE)	1 (4.3%)	1 (4.3%)	1.00
DEATH	1 (4.3%)	0	1.00
NICU LENGTH OF STAY, d	18.4±11.4	6.7±2.7	< .001[a]

MEANS AND SDS ARE PRESENTED FOR CONTINUOUS VARIABLES; COUNTS AND PERCENTAGES, ODDS, RATIOS, AND 95% CONFIDENCE INTERVALS ARE PRESENTED FOR CATEGORICAL VARIABLES.
CSF, CEREBROSPINAL FLUID; FHR, FETAL HEART RATE; HIE, HYPOXIC-ISCHEMIC ENCEPHALOPATHY; NICU, NEONATAL INTENSIVE CARE UNIT.

[a] STATISTICAL SIGNIFICANCE WITH P< .05.

Ennen. Glial fibrillary acidic protein in hypoxic-ischemic encephalopathy. Am J Obstet Gynecol 2011.

FIG. 18
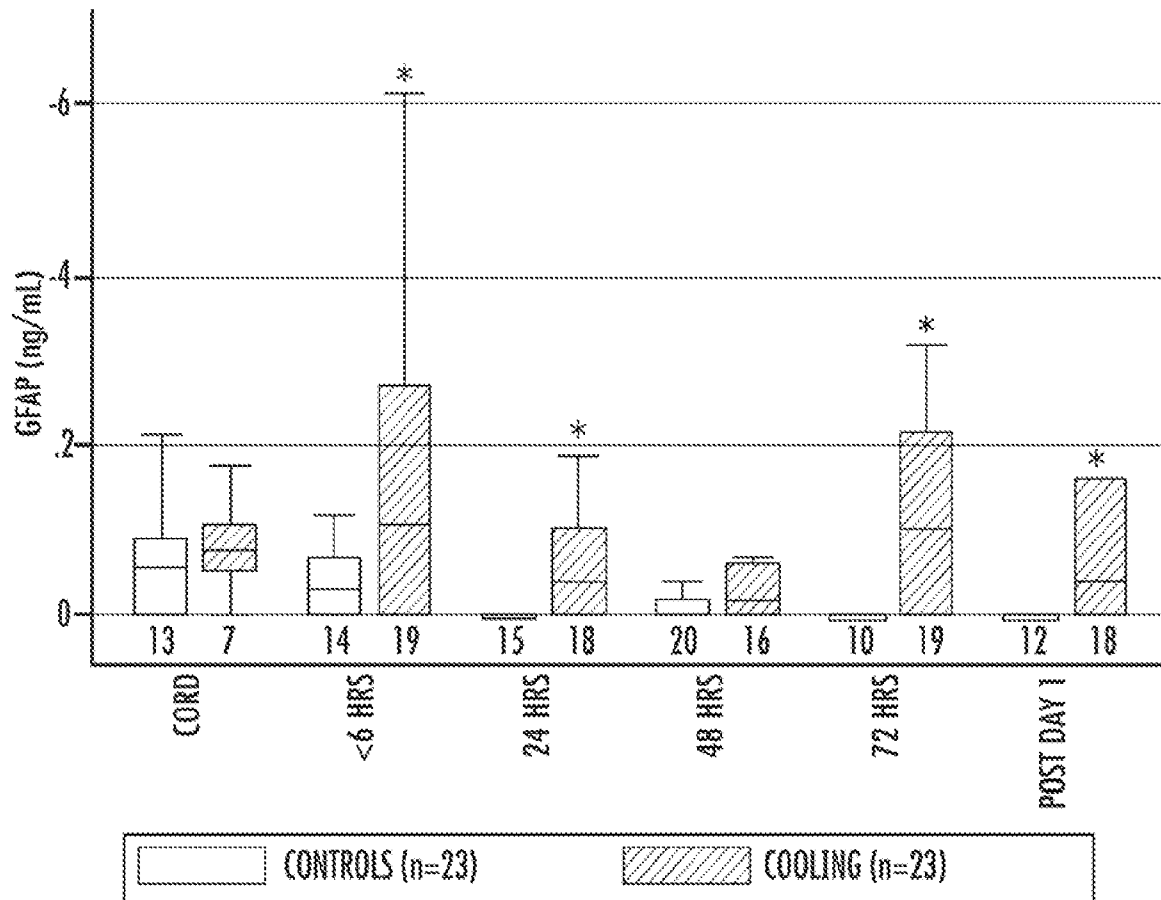
FIG. 19

CHARACTERISTICS FOR NEONATES TREATED WITH COOLING			
CHARACTERISTIC	ABNORMAL MRI (n=8)	NORMAL MRI (n=15)	P VALUE
GESTATIONAL AGE, WKS	37.9±1.3	39.0±1.4	.073
BIRTHWEIGHT, g	2807±643	3069±625	.353
NULLIPAROUS	6(75%)	8(53.3%)	.400
CESAREAN DELIVERY	7(87.5%)	8(53.3%)	.176
IUGR	0	4(26.7%)	.257
CLINICAL CHORIOAMNIONITIS	1(12.5%)	2(13.3%)	1.00
NONREASSURING FHR TRACING	5(62.5%)	3(20%)	.400
ABRUPTION	6(75%)	2(13.3%)	.006[a]
pH	6.85±0.20	6.95±0.19	.322
BASE DEFICIT, mM	19.6±4.3	13.8±6.9	.104
CORD pH <7.0 AND BASE DEFICIT >12	7/7(100%)	9/12 (75%)	.263

MEANS AND SDS ARE PRESENTED FOR CONTINUOUS VARIABLES; COUNTS AND PERCENTAGES ARE PRESENTED FOR CATEGORICAL VARIABLES. FHR, FETAL HEART RATE; HIE, HYPOXIC-ISCHEMIC ENCEPHALOPATHY; IUGR, INTRAUTERINE GROWTH RESTRICTION; MRI, MAGNETIC RESONANCE IMAGING.

[a] STATISTICAL SIGNIFICANCE WITH P< .05.

Ennen. Glial fibrillary acidic protein in hypoxic-ischemic encephalopathy. Am J Obstet Gynecol 2011.

FIG. 20
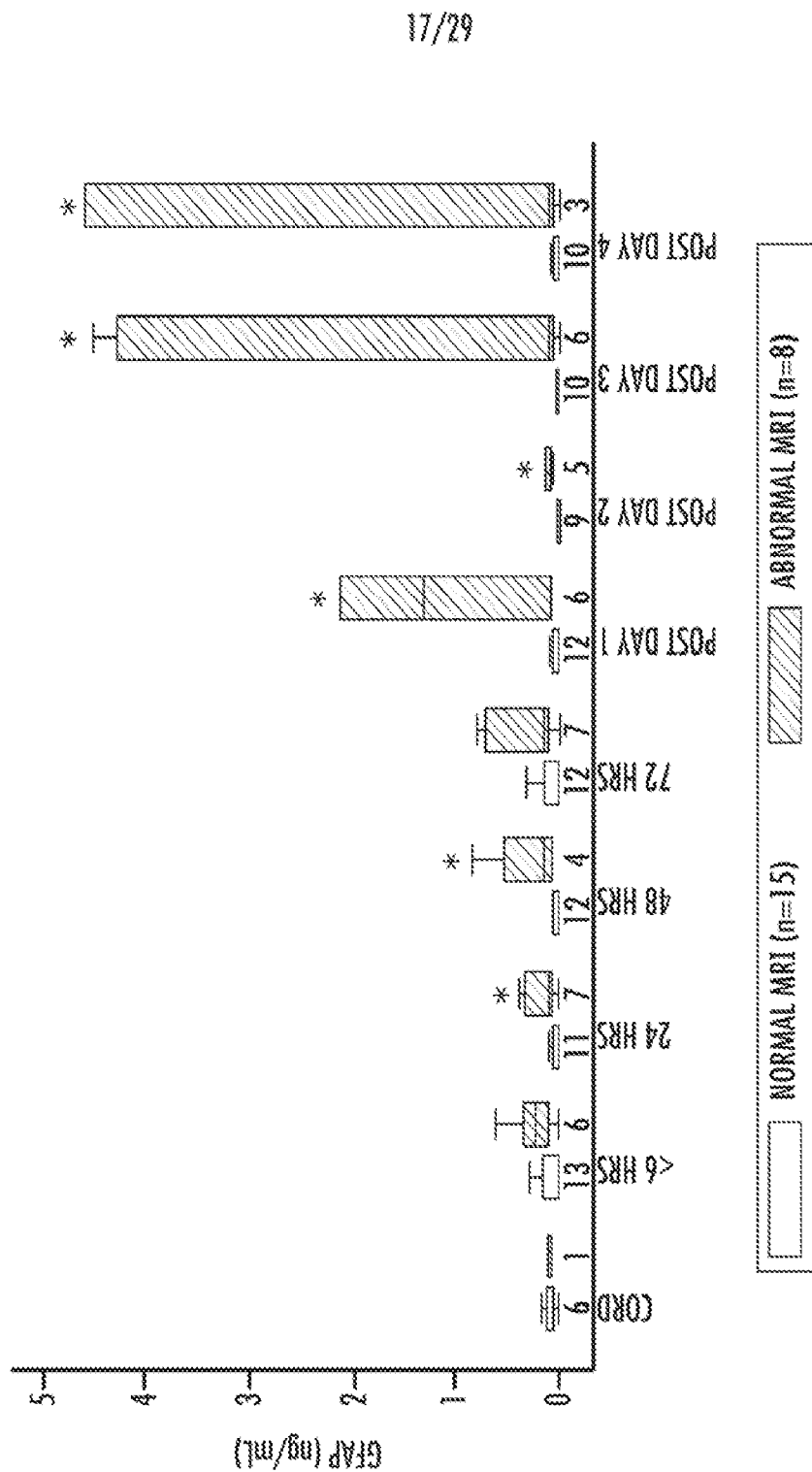
FIG. 21

GFAP LEVELS AND NEUROLOGICAL OUTCOMES

VARIABLE	n	AGE, d	GFAP ON NICU ADMISSION, ng/mL
ABNORMAL MRI (n=8)			
DEATH	1	7	.61
G-TUBEFEEDING	5	114±81	0.26±0.10
FULL ORAL FEEDING	2	17±9	0.04±0.06[a]
NORMAL MRI (n=15)			
DEATH	—	—	—
G-TUBEFEEDING	—	—	—
FULL ORAL FEEDING	15	9±5	0.08±0.09

GFAP, glial fibrillary acidic protein, HIE, HYPOXIC-ISCHEMIC ENCEPHALOPATHY; MRI, MAGNETIC RESONANCE IMAGING; NICU, intensive care unit.

[a]P= .03 compared with normal MRI.

Ennen. Glial fibrillary acidic protein in hypoxic-ischemic encephalopathy. AmJObstetGynecol 2011.

FIG. 22

| log(e) log(I) m+h delta z sequence | validate | studio
5737 -8.6 4.88 2195.0615 0.0020 2 [21]IPESGGDNSVFDIFELTGAAR[41]

ALLEN-A12.mzXML scan 5737 (CHARGE 2)

I·P·E·S·G·G·D·N·S·V·F·D·I·F·E·L·T·G·A·A·R

view: Δ ▲

m/z

ERROR (Da)
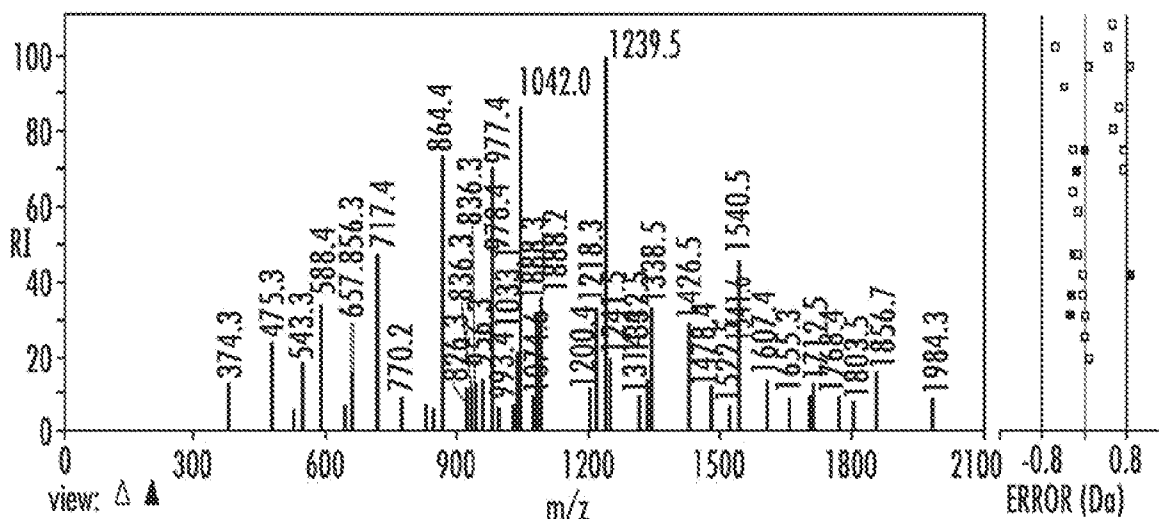
bond	+1y	+1y-17	+1y-18	+1b	+1b-17	+1b-18
I₁	+2 2081.977	2064.951	2063.967	114.091	97.065	96.081
P₂	+1,2 1984.925	1967.898	1966.914	211.144	194.118	193.134
E₃	+2 1855.882	1838.855	1837.871	340.187	323.160	322.176
S₄	1768.850	1751.823	1750.839	427.219	410.192	409.208
G₅	1711.829	1694.802	1693.818	484.240	467.214	466.230
G₆	1654.807	1637.781	1636.797	541.262	542.235	523.251
D₇	+1,2 1539.780	1522.754	1521.770	656.289	639.262	638.278
N₈	1425.737	1408.711	1407.727	770.332	753.305	752.321
S₉	1338.705	1321.679	1320.695	857.364	840.337	839.353
V₁₀	1239.637	1222.610	1221.626	956.432	939.405	938.421
F₁₁	1092.568	1075.542	1074.558	1103.500	1086.474	1085.490
D₁₂	977.541	960.515	959.531	1218.527	1201.501	1200.517
I₁₃	864.457	847.431	846.447	1331.611	1314.585	1313.601
F₁₄	717.389	700.362	699.378	1478.680	1461.653	1460.669
E₁₅	588.346	571.320	570.336	1607.722	1590.696	1589.712
L₁₆	475.262	458.236	457.252	1720.806	1703.780	1702.796
T₁₇	374.215	357.188	356.204	1821.854	1804.828	1803.844
G₁₈	317.193	300.167	299.183	1878.876	1861.849	1860.865
A₁₉	246.156	229.130	228.145	1949.913	1932.886	1931.902
A₂₀	175.119	158.092	157.108	2020.950	2003.923	2002.939

FIG. 23

log(e) log(I)   m+h   delta   z   sequence |validate|studio
3760 -4.3   4.69 1889.8148 0.0033 2   [119]SLTEEAENWGDGEPNNK[135]
78A6.mzXML scan 3760 (CHARGE 2)

S·L·T·E·E·A·E*N·W·G·D*·G·E·P·N·N·K
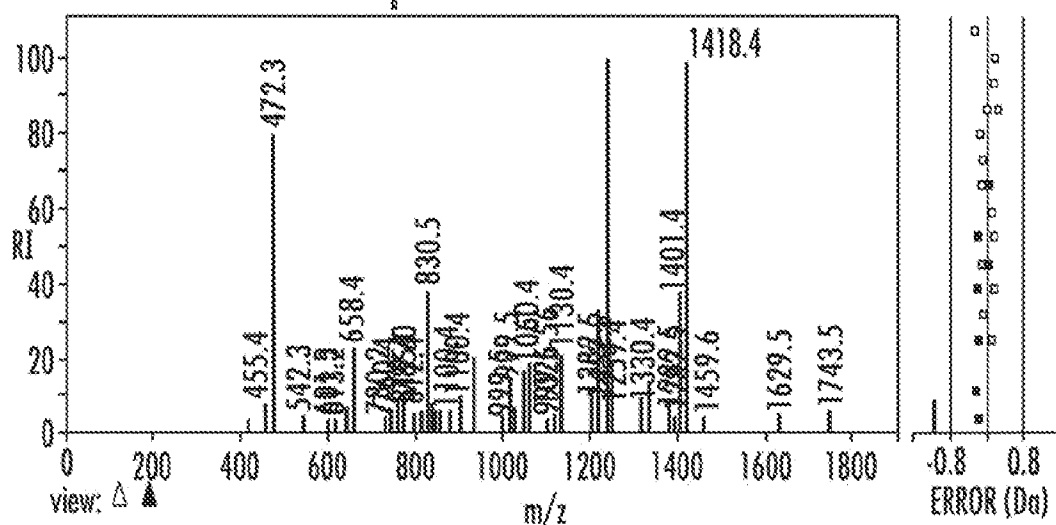
bond	+1y	+1y-17	+1y-18	+1b	+1b-17	+1b-18
S₁	+2 1802.783	1785.756	1784.772	88.039	71.013	70.029
L₂	+2 1689.699	1672.672	1671.688	201.123	184.097	183.113
T₃	+2 1588.651	1571.624	1570.640	302.171	285.144	284.160
E₄	+1,2 1459.608	1442.582	1441.598	431.214	414.187	413.203
E₅	1330.566	1313.539	1312.555	560.256	543.230	542.246
A₆	1259.529	1242.502	1241.518	631.293	614.267	613.283
E₇	1130.486	1113.460	1112.476	760.336	743.309	742.325
N₈	1016.443	999.417	998.433	874.379	857.352	856.368
W₉	830.364	813.337	812.353	1060.458	1043.432	1042.448
G₁₀	773.342	756.316	755.332	1117.480	1100.453	1099.469
D₁₁	658.315	641.289	640.305	1232.507	1215.480	1214.496
G₁₂	601.294	584.267	583.283	1289.528	1272.501	1271.517
E₁₃	472.251	455.225	454.241	1418.571	1401.544	1400.560
P₁₄	375.199	358.172	357.188	1515.623	1498.597	1497.613
N₁₅	261.156	244.129	243.145	1629.666	1612.640	1611.656
N₁₆	147.113	130.086	129.102	1743.709	1726.683	1725.699

FIG. 24

FIG. 25

FIG. 26

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FIG. 27

FIG. 28

FIG. 29

FIG. 30

188 Kd

98 Kd

62 Kd

49 Kd

38 Kd

28 Kd

17 Kd

14 Kd

6 Kd

His-NRGN →

FIG. 31

PLOT: STANDARD

FIG. 32

FIG. 33

FIG. 34