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(54) **BIOMARKER DETECTION PROCESS AND ASSAY OF NEUROLOGICAL CONDITION**

VERFAHREN FÜR DEN NACHWEIS VON BIOMARKERN UND TEST FÜR NEUROLOGISCHE ERKRANKUNG

PROCÉDÉ DE DÉTECTION DE BIOMARQUEURS ET TEST D'ÉTAT NEUROLOGIQUE

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Description

GOVERNMENTAL SUPPORT

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CROSS REFERENCE TO RELATED APPLICATIONS

10 **[0002]** This application claims priority to US Provisional Application No. 61/188,554 filed August 11, 2008; US Provisional Application No. 61/097,622 filed September 17, 2008; U.S. Provisional Application No. 61/218,727 filed June 19, 2009; and US Provisional Application No. 61/271,135 filed July 18, 2009.

FIELD OF THE INVENTION

15 **[0003]** The present invention in general relates to determination of neurological condition of an individual, wherein said neurological condition is selected from traumatic brain injury and stroke, and in particular to measuring the quantity of a neuroprotective biomarker such as glial fibrillary acidic protein (GFAP) in concert with another biomarker of neurological condition.

20 BACKGROUND OF THE INVENTION

[0004] The field of clinical neurology remains frustrated by the recognition that secondary injury to a central nervous system tissue associated with physiologic response to the initial insult could be lessened if only the initial insult could be rapidly diagnosed or in the case of a progressive disorder before stress on central nervous system tissues reached a preselected threshold. Traumatic, ischemic, and neurotoxic chemical insult, along with generic disorders, all present the prospect of brain damage. While the diagnosis of severe forms of each of these causes of brain damage is straightforward through clinical response testing, computed tomography (CT), and magnetic resonance imaging (MRI), the imaging diagnostics are limited by both the high cost of spectroscopic imaging and long diagnostic time. The clinical response testing of incapacitated individuals is of limited value and often precludes a nuanced diagnosis. Additionally, owing to the limitations of existing diagnostics, situations arise wherein a subject experiences a stress to their neurological condition but are often unaware that damage has occurred or fail seek treatment as the subtle symptoms often quickly resolve. The lack of treatment of these mild to moderate challenges to neurologic condition of a subject can have a cumulative effect or otherwise result in a severe brain damage event, either of which have a poor clinical prognosis.

35 **[0005]** In order to overcome the limitations associated with spectroscopic and clinical response diagnosis of neurological condition, there is increasing attention on the use of biomarkers as internal indicators of change to molecular or cellular level health condition of a subject. As biomarker detection uses a sample obtained from a subject, typically cerebrospinal fluid, blood, or plasma, and detects the biomarkers in that sample, biomarker detection holds the prospect of inexpensive, rapid, and objective measurement of neurological condition. The attainment of rapid and objective indicators of neurological condition allows one to determine severity of a non-normal brain condition with a previously unrealized degree of objectivity, predict outcome, guide therapy of the condition, as well as monitor subject responsiveness and recovery. Additionally, such information as obtained from numerous subjects allows one to gain a degree of insight into the mechanism of brain injury.

45 **[0006]** A number of biomarkers have been identified as being associated with severe traumatic brain injury as is often seen in vehicle collision and combat wounded subjects. These biomarkers included spectrin breakdown products such as SBDP150, SBDP150i, SBDP145 (calpain mediated acute neural necrosis), SBDP120 (caspase mediated delayed neural apoptosis), UCH-L1 (neuronal cell body damage marker), and MAP2 dendritic cell injury associated marker. The nature of these biomarkers is detailed in U.S. Patents 7,291,710 and 7,396,654.

50 **[0007]** Glial Fibrillary Acidic Protein (GFAP), a member of the cytoskeletal protein family, is the principal 8-9 nanometer intermediate filament of glial cells such as mature astrocytes of the central nervous system (CNS). GFAP is a monomeric molecule with a molecular mass between 40 and 53 kDa and an isoelectric point between 5.7 and 5.8. GFAP is highly brain specific protein that is not found outside the CNS. GFAP is released into the blood and CSF soon after brain injury. In the CNS following injury, either as a result of trauma, disease, genetic disorders, or chemical insult, astrocytes become reactive in a way that is characterized by rapid synthesis of GFAP termed astrogliosis or gliosis. However, GFAP normally increases with age and there is a wide variation in the concentration and metabolic turnover of GFAP in brain tissue.

55 **[0008]** Thus, there exists a need for a process and an assay for providing improved measurement of neurological condition through the quantification of a first biomarker such as GFAP in combination with another biomarker associated with neurological condition.

SUMMARY OF THE INVENTION

[0009] A process for determining the neurological condition of a subject, wherein said neurological condition is selected from traumatic brain injury and stroke includes measuring a sample obtained from the subject at a first time for a quantity of a first biomarker and a quantity of at least one additional neuroactive biomarker, wherein said first biomarker is GFAP and said at least one additional neuroactive biomarker is UCH-L1. Through comparison of the quantity of the first biomarker and the quantity of the at least one additional neuroactive biomarker to normal levels for each biomarker, the neurological condition of the subject is determined. When the subject have been exposed to an event that could cause mild traumatic brain injury and moderate traumatic brain injury, a process of measuring UCH-L1 and GFAP, such injuries have detection cutoffs for UCH-L1 and GFAP in serum of 0.39 nanograms per milliliter (ng/ml) and 1.4ng/ml, respectively.

[0010] An assay for determining the neurological condition of a subject is also provided. The assay includes: (a) a substrate for holding a biological sample isolated from a subject (b) a first biomarker specifically binding agent wherein a first biomarker is GFAP (c) a binding agent specific for another neuroactive biomarker wherein said another neuroactive biomarker is UCH-L1; and (d) printed instructions for reacting the first biomarker specific agent with a first portion of the sample so as to detect an amount of said first biomarker and reacting said at least one additional neuroactive biomarker specific agent with a second portion of the sample and the at least one additional neuroactive biomarker in the sample so as to detect an amount of said at least one additional neuroactive biomarker for relation to the condition of the subject or cells derived the subject.

[0011] A process for determining if a subject has suffered mild traumatic brain injury or moderate traumatic brain injury in an event comprising: measuring by ELISA a biological sample obtained from said subject for a quantity of GFAP, wherein said biological sample is whole blood, serum or plasma; and comparing the measured quantity of said GFAP to normal levels of GFAP in a control group who had not experienced any form of head injury, wherein if the measured quantity of GFAP is between 0.082 and 0.492 nanograms per milliliter, the subject has suffered mild traumatic brain injury or moderate brain injury in the event.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012]

Fig. 1 represents quantitative western blotting of UCH-L1 in rat CSF following MCAO;

Fig. 2 represents UCH-L1 levels in CSF in sham and CCI treated subjects;

Fig. 3 represents UCH-L1 levels in CSF following sham, mild MCAO challenge, and severe MCAO challenge;

Fig. 4 represents UCH-L1 levels in serum following sham or CCI at various timepoints;

Fig. 5 represents UCH-L1 levels in serum following sham, mild MCAO challenge, and severe MCAO challenge;

Fig. 6 represents SBDP145 levels in CSF and serum following sham, mild MCAO challenge, and severe MCAO challenge;

Fig. 7 represents SBDP120 levels in CSF and serum following sham, mild MCAO challenge, and severe MCAO challenge;

Fig. 8 represents MAP2 elevation in CSF and serum following sham, mild MCAO challenge, and severe MCAO challenge;

Fig. 9 are bar graphs of GFAP and other biomarkers for human control and severe TBI subjects from CSF samples; **Fig. 10** are bar graphs of GFAP and other biomarkers for human control and severe TBI subjects of Fig. 1 from serum samples;

Fig. 11 are bar graphs of GFAP and other biomarkers for human control and severe TBI subjects summarizing the data of FIGS. 9 and 10;

Fig. 12 are plots of arterial blood pressure (MABP), intracranial pressure (ICP) and cerebral perfusion pressure (CPP) for a single human subject of traumatic brain injury as a function of time;

Fig. 13 are plots of inventive biomarkers from CSF and serum samples from the single human subject of traumatic brain injury of FIG. 12 as a function of time;

Fig. 14 are plots of inventive biomarkers from CSF and serum samples from another individual human subject of traumatic brain injury as a function of time;

Fig. 15 are plots of UCH-L1 amounts being present in CSF and serum post severe traumatic brain injury in a mouse subject;

Fig. 16 are bar graphs of GFAP concentration for controls, as well as individuals in the mild/moderate traumatic brain injury cohort as a function of CT scan results upon admission and 24 hours thereafter;

Fig. 17 are bar graphs of parallel assays for UCH-L1 biomarker from the samples used for FIG. 16;

Fig. 18 are bar graphs showing the concentration of UCH-L1 and GFAP as well as a biomarker not selected for diagnosis of neurological condition, S100 beta, provided as a function of injury magnitude between control, mild,

and moderate traumatic brain injury;

Fig. 19 are bar graphs showing the concentration of the same markers as depicted in FIG. 18 with respect to initial evidence upon hospital admission as to lesions in tomography scans;

Fig. 20 represents biomarker levels in human subjects with varying types of brain injury;

Fig. 21 are plots that represent ROC analysis of UCH-L1, GFAP and SBDP145 in human CSF (severe TBI vs. Control A) First 24 hours post-injury;

Fig. 22 is a plot that represent ROC analysis of UCH-L1 and GFAP in human CSF (mild TBI vs. normal Controls) a mean of 3h35' with a range 15'-14h35 post-injury.

Fig. 23 are bar graphs of showing the elevation of brain injury biomarkers (GFAP, UCH-L1 and MAP2) in plasma from stroke patients.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0013] The present invention has utility in the diagnosis and management of abnormal neurological condition, wherein said neurological condition is selected from traumatic brain injury and stroke. Through the measurement of a biomarker such as GFAP from a subject in combination with values obtained for an additional neuroactive biomarker, a determination of subject neurological condition is provided with greater specificity than previously attainable. The present description is directed toward a first biomarker of GFAP. It is appreciated that the invention encompasses several other additional biomarkers illustratively including UCH-L1, NSE, MAP2, and SBDP, wherein at least one additional biomarker is UCH-L1. Surprisingly, by combining the detection of more than one biomarker, a synergistic result is achieved. Illustratively, combining the detection of two neuroactive biomarkers such as UCH-L1 and GFAP provides sensitive detection that is unexpectedly able to discern the level and severity of an abnormal neurological condition in a subject.

[0014] The present invention provides for the detection of a neurological condition in a subject, wherein said neurological condition is selected from traumatic brain injury an stroke. As such, it is a further object of the present invention to provide a means for detecting or diagnosing an abnormal neurological condition in a subject.

[0015] The present invention also provides an assay for detecting or diagnosing the neurological condition of a subject.

[0016] For purposes of the subject invention, brain injury is divided into two levels, mild traumatic brain injury (MTBI), and traumatic brain injury (TBI). An intermediate level of moderate TBI is also referred to herein. The spectrum between MTBI and extending through moderate TBI is also referred to synonymously mild to moderate TBI or by the abbreviation MMTBI. TBI is defined as an injury that correlates with a two-fold increase or greater of two-fold decrease or greater in molecular marker levels or activities. MTBI is defined and an injury that correlates with less than a two-fold increase or two-fold decrease in molecular marker levels or activities.

[0017] An inventive process preferably includes determining the neurological condition of a subject by assaying a sample derived from a subject at a first time for the presence of a first biomarker. A biomarker is a cell, protein, nucleic acid, steroid, fatty acid, metabolite, or other differentiator useful for measurement of biological activity or response. Biomarkers operable herein illustratively include: ubiquitin carboxyl-terminal esterase, ubiquitin carboxy-terminal hydrolyase, spectrin breakdown product(s), a neuronally-localized intracellular protein, MAP-tau, C-tau, MAP2, poly (ADP-ribose) polymerase (PARP), collapsin response mediator protein, Annexin A11, Aldehyde dehydrogenase family 7, Cofilin 1, Profilin 1, α -Enolase (non-neural enolase), Enolase 1 protein, Glyceraldehyde-3-phosphate dehydrogenase, Hexokinase 1, Aconitase 2, Acetyl-CoA synthetase 2, Neuronal protein 22, Phosphoglycerate kinase 2, Phosphoglycerate kinase 1, Hsc70-ps1, Glutamate dehydrogenase 1, Aldolase A, Aldolase C, fructose-biphosphate, Dimethylarginine dimethylaminohydrolase 1, Microtubule-associated protein 2, Carbonic anhydrase, ADP-ribosylation factor 3, Transferrin, Liver regeneration-related protein, Hemoglobin α -chain, Hemoglobin β chain, Liver regeneration-related protein, Fetuin β , 3-Oxoacid-CoA transferase, Malate dehydrogenase 1, NAD (soluble), Lactate dehydrogenase B, Malate dehydrogenase, Carboxylesterase E1 precursor, Serine protease inhibitor α 1, Haptoglobin, Ubiquitin carboxyl-terminal hydrolase L1, Serine protease inhibitor 2a, T-kininogen, α 1 major acute phase protein, Albumin, α 1 major acute phase protein prepeptide, Murinoglobulin 1 homolog, Group-specific component protein, Guanosine diphosphate dissociation inhibitor 1, Collapsin response mediator protein 2, Murinoglobulin 1 homolog, Ferroxidase, Ceruloplasmin, Spectrin α -chain, brain, C-reactive protein, Brain creatine kinase, Proteasome subunit α -type 7, 14-3-3 protein, Synaptotagmin, subtypes thereof, fragments thereof, breakdown products thereof, or combinations thereof. Other potential biomarkers illustratively include those identified by Kobeissy, FH, et al, Molecular & Cellular Proteomics, 2006; 5:1887-1898, the contents of which are incorporated herein by reference, or others known in the art.

[0018] A first biomarker is a neuroactive biomarker GFAP. Illustrative examples of neuroactive biomarkers include GFAP, ubiquitin carboxyl-terminal esterase L1 (UCH-L1), Neuron specific enolase (NSE), spectrin breakdown products (SBDP), preferably SBDP150, SBDP150i SBDP145, SBDP120, S100 calcium binding protein B (S100b), microtubule associated proteins (MAP), preferably MAP2, MAP1, MAP3, MAP4, MAP5, myelin basic protein (MBP), Tau, Neurofilament protein (NF), Cannabinoid Receptor (CB), CAM proteins, Synaptic protein, collapsin response mediator proteins (CRMP), inducible nitric oxide synthase (iNOS), Neuronal Nuclei protein (NeuN), 2',3'-cyclic nucleotide-3'-phosphohy-

drolase (CNPase), Neuroserpin, alpha-internexin, microtubule-associated protein 1 light chain 3 (LC3), Neurofascin, the glutamate transporters (EAAT), Nestin, Cortin-1, 2', , and BIII-Tubulin.

[0019] The inventive process also includes assaying the sample for at least one additional neuroactive biomarker, wherein said at least one additional neuroactive biomarker is UCH-L1. Any of the aforementioned inventive biomarkers are operable as an additional neuroactive biomarker. Any number of biomarkers can be detected such as 2, 3, 4, 5, 6, 7, 8, 9, 10, or more. Detection can be either simultaneous or sequential and may be from the same biological sample or from multiple samples from the same or different subjects. Preferably, detection of multiple biomarkers is in the same assay chamber. The inventive process further includes comparing the quantity of the first biomarker and the quantity of the at least one additional neuroactive biomarker to normal levels of each of the first biomarker and the one additional neuroactive biomarker to determine the neurological condition of the subject.

[0020] In a preferred embodiment a biomarker is GFAP. GFAP is associated with glial cells such as astrocytes. Preferably, an additional neuroactive biomarker is associated with the health of a different type of cell associated with neural function. For example, CNPase is found in the myelin of the central nervous system, and NSE is found primarily in neurons. More preferably, the other cell type is an axon, neuron, or dendrite.

[0021] In another preferred embodiment, especially for MBTI and MMTBI, is UCH-L1 in combination with other biomarkers such as GFAP and MAP2.

[0022] It is appreciated however, that multiple biomarkers may be predictors of different modes or types of damage to the same cell type. Through the use of an inventive assay inclusive of biomarkers associated with glial cells as well as at least one other type of neural cell, the type of neural cells being stressed or killed as well as quantification of neurological condition results provides rapid and robust diagnosis of traumatic brain injury type. Measuring GFAP along with at least one additional neuroactive biomarker and comparing the quantity of GFAP and the additional biomarker to normal levels of the markers provides a determination of subject neurological condition.

[0023] Preferably, specific biomarker levels that when measured in concert with GFAP afford superior evaluation of subject neurological condition include SBDP 150, SBDP150i, a combination of SBDP145 (calpain mediated acute neural necrosis) and SBDP120 (caspase mediated delayed neural apoptosis), UCH-L1 (neuronal cell body damage marker), and MAP2. This is noted to be of particular value in measuring MMTBI and screening drug candidates or other neural cell stressor compounds with cell cultures.

[0024] A sample is preferably a biological sample. Preferred examples of biological samples are illustratively cells, tissues, cerebral spinal fluid (CSF), artificial CSF, whole blood, serum, plasma, cytosolic fluid, urine, feces, stomach fluids, digestive fluids, saliva, nasal or other airway fluid, vaginal fluids, semen, buffered saline, saline, water, or other biological fluid recognized in the art. Most preferably, a biological sample is CSF or blood serum. It is appreciated that two or more separate biological samples are optionally assayed to elucidate the neurological condition of the subject.

[0025] In addition to increased cell expression, biomarkers also appear in biological fluids in communication with injured cells. Obtaining biological fluids such as cerebrospinal fluid (CSF), blood, plasma, serum, saliva and urine, from a subject is typically much less invasive and traumatizing than obtaining a solid tissue biopsy sample. Thus, samples that are biological fluids are preferred for use in the invention. CSF, in particular, is preferred for detecting nerve damage in a subject as it is in immediate contact with the nervous system and is readily obtainable. Serum is a preferred biological sample as it is easily obtainable and presents much less risk of further injury or side-effect to a donating subject.

[0026] To provide correlations between neurological condition and measured quantities of GFAP and other neuroactive biomarkers, samples of CSF or serum are collected from subjects with the samples being subjected to measurement of GFAP as well as other neuroactive biomarkers. The subjects vary in neurological condition. Detected levels of GFAP and other neuroactive biomarkers are optionally then correlated with CT scan results as well as GCS scoring. Based on these results, an inventive assay is developed and validated (Lee et al., *Pharmacological Research* 23:312-328, 2006). It is appreciated that GFAP and other neuroactive biomarkers, in addition to being obtained from CSF and serum, are also readily obtained from blood, plasma, saliva, urine, as well as solid tissue biopsy. While CSF is a preferred sampling fluid owing to direct contact with the nervous system, it is appreciated that other biological fluids have advantages in being sampled for other purposes and therefore allow for inventive determination of neurological condition as part of a battery of tests performed on a single sample such as blood, plasma, serum, saliva or urine.

[0027] A biological sample is obtained from a subject by conventional techniques. For example, CSF is preferably obtained by lumbar puncture. Blood is preferably obtained by venipuncture, while plasma and serum are obtained by fractionating whole blood according to known methods. Surgical techniques for obtaining solid tissue samples are well known in the art. For example, methods for obtaining a nervous system tissue sample are described in standard neurosurgery texts such as *Atlas of Neurosurgery: Basic Approaches to Cranial and Vascular Procedures*, by F. Meyer, Churchill Livingstone, 1999; *Stereotactic and Image Directed Surgery of Brain Tumors*, 1st ed., by David G. T. Thomas, WB Saunders Co., 1993; and *Cranial Microsurgery: Approaches and Techniques*, by L. N. Sekhar and E. De Oliveira, 1st ed., Thieme Medical Publishing, 1999. Methods for obtaining and analyzing brain tissue are also described in Belay et al., *Arch. Neurol.* 58: 1673-1678 (2001); and Seijo et al., *J. Clin. Microbiol.* 38: 3892-3895 (2000).

[0028] After insult, nerve cells in *in vitro* culture or *in situ* in a subject express altered levels or activities of one or more

proteins than do such cells not subjected to the insult. Thus, samples that contain nerve cells, e.g., a biopsy of a central nervous system or peripheral nervous system tissue are illustratively suitable biological samples for use in the invention. In addition to nerve cells, however, other cells express illustratively all-spectrin including, for example, cardiomyocytes, myocytes in skeletal muscles, hepatocytes, kidney cells and cells in testis. A biological sample including such cells or fluid secreted from these cells might also be used in an adaptation of the inventive methods to determine and/or characterize an injury to such non-nerve cells.

[0029] A subject illustratively includes a dog, a cat, a horse, a cow, a pig, a sheep, a goat, a chicken, non-human primate, a human, a rat, and a mouse. Subjects who most benefit from the present invention are those suspected of having or at risk for developing abnormal neurological conditions, such as victims of brain injury caused by traumatic insults (e.g., gunshot wounds, automobile accidents, sports accidents, shaken baby syndrome), ischemic events (e.g., stroke, cerebral hemorrhage, cardiac arrest), neurodegenerative disorders (such as Alzheimer's, Huntington's, and Parkinson's diseases; prion-related disease; other forms of dementia), epilepsy, substance abuse (e.g., from amphetamines, Ecstasy/MDMA, or ethanol), and peripheral nervous system pathologies such as diabetic neuropathy, chemotherapy-induced neuropathy and neuropathic pain.

[0030] Baseline levels of several biomarkers are those levels obtained in the target biological sample in the species of desired subject in the absence of a known neurological condition. These levels need not be expressed in hard concentrations, but may instead be known from parallel control experiments and expressed in terms of fluorescent units, density units, and the like. Typically, in the absence of a neurological condition SBDPs are present in biological samples at a negligible amount. However, UCH-L1 is a highly abundant protein in neurons. Determining the baseline levels of UCH-L1 in neurons of particular species is well within the skill of the art. Similarly, determining the concentration of baseline levels of MAP2, GFAP, NSE, or other biomarker is well within the skill of the art.

[0031] As used herein the term "diagnosing" means recognizing the presence or absence of a neurological or other condition such as an injury or disease. Diagnosing is optionally referred to as the result of an assay wherein a particular ratio or level of a biomarker is detected or is absent.

[0032] As used herein a "ratio" is either a positive ratio wherein the level of the target is greater than the target in a second sample or relative to a known or recognized baseline level of the same target. A negative ratio describes the level of the target as lower than the target in a second sample or relative to a known or recognized baseline level of the same target. A neutral ratio describes no observed change in target biomarker.

[0033] 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. Injury illustratively includes a physical, mechanical, chemical, biological, functional, infectious, or other modulator of cellular or molecular characteristics. An event is illustratively, 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 events that are encompassed by the terms injury or event.

[0034] An injury is optionally a physical event such as a percussive impact. An impact is the like of a percussive injury such as resulting to a blow to the head that either leaves the cranial structure intact or results in breach thereof. Experimentally, several impact methods are used illustratively including controlled cortical impact (CCI) at a 1.6 mm depression depth, equivalent to severe TBI in human. This method is described in detail by Cox, CD, et al., J Neurotrauma, 2008; 25(11):1355-65. It is appreciated that other experimental methods producing impact trauma are similarly operable.

[0035] TBI may also result from stroke. Ischemic stroke is optionally modeled by middle cerebral artery occlusion (MCAO) in rodents. UCH-L1 protein levels, for example, are increased following mild MCAO which is further increased following severe MCAO challenge. Mild MCAO challenge may result in an increase of protein levels within two hours that is transient and returns to control levels within 24 hours. In contrast, severe MCAO challenge results in an increase in protein levels within two hours following injury and may be much more persistent demonstrating statistically significant levels out to 72 hours or more.

[0036] An exemplary process for detecting the presence or absence of GFAP and one or more other neuroactive biomarkers in a biological sample involves obtaining a biological sample from a subject, such as a human, contacting the biological sample with a compound or an agent capable of detecting of the marker being analyzed, illustratively including an antibody or aptamer, and analyzing binding of the compound or agent to the sample after washing. Those samples having specifically bound compound or agent express the marker being analyzed.

[0037] An inventive process can be used to detect GFAP and one or more other neuroactive biomarkers in a biological sample *in vitro*, as well as *in vivo*. The quantity of GFAP and one or more other neuroactive biomarkers in a sample is compared with appropriate controls such as a first sample known to express detectable levels of the marker being analyzed (positive control) and a second sample known to not express detectable levels of the marker being analyzed (a negative control). For example, *in vitro* techniques for detection of a marker illustratively include enzyme linked immunosorbent assays (ELISAs), radioimmuno assay, radioassay, western blot, Southern blot, northern blot, immunoprecipitation, immunofluorescence, mass spectrometry, RT-PCR, PCR, liquid chromatography, high performance liquid chromatography, enzyme activity assay, cellular assay, positron emission tomography, mass spectroscopy, combina-

tions thereof, or other technique known in the art.. Furthermore, *in vivo* techniques for detection of a marker include introducing a labeled agent that specifically binds the marker into a biological sample or test subject. For example, the agent can be labeled with a radioactive marker whose presence and location in a biological sample or test subject can be detected by standard imaging techniques. Optionally, the first biomarker specifically binding agent and the agent specifically binding at least one additional neuroactive biomarker are both bound to a substrate. It is appreciated that a bound agent assay is readily formed with the agents bound with spatial overlap, with detection occurring through discernibly different detection for first biomarker and each of at least one additional neuroactive biomarkers. A color intensity based quantification of each of the spatially overlapping bound biomarkers is representative of such techniques.

[0038] Any suitable molecule that can specifically bind GFAP and any suitable molecule that specifically binds one or more other neuroactive biomarkers are operative in the invention to achieve a synergistic assay. A preferred agent for detecting GFAP or the one or more other neuroactive biomarkers is an antibody capable of binding to the biomarker being analyzed. Preferably, an antibody is conjugated with a detectable label. Such antibodies can be polyclonal or monoclonal. An intact antibody, a fragment thereof (e.g., Fab or F(ab')₂), or an engineered variant thereof (e.g., sFv) can also be used. Such antibodies can be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. Antibodies for numerous inventive biomarkers are available from vendors known to one of skill in the art. Illustratively, antibodies directed to inventive biomarkers are available from Santa Cruz Biotechnology (Santa Cruz, CA). Exemplary antibodies operative herein to detect a first biomarker include anti-GFAP antibody, anti-UCH-L1 antibody, anti-NSE antibody, anti-MAP2 antibody, or an anti-SBDP antibody. Other biomarkers to be targeted as part of an inventive assay different from the first biomarker include GFAP, NSE, SBDP, SBDP150, SBDP145, SBDP120, S100b, MAP2, MAP1, MAP3, MAP4, MAP5, MBP, Tau, Neurofilament protein (NF), Cannabinoid Receptor CB, CAM, Synaptic protein, CRMP, iNOS, NeuN, CSPase, Neuroserpin, alpha-internexin, LC3, Neurofascin, EAAT, Nestin, Cortin-1, or BIII-Tubulin

[0039] An antibody is optionally labeled. A person of ordinary skill in the art recognizes numerous labels operable herein. Labels and labeling kits are commercially available optionally from Invitrogen Corp, Carlsbad, CA. Labels illustratively include, fluorescent labels, biotin, peroxidase, radionucleotides, or other label known in the art. Alternatively, a detection species of another antibody or other compound known to the art is used as form detection of a biomarker bound by an antibody.

[0040] Antibody-based assays are preferred for analyzing a biological sample for the presence of GFAP and one or more other neuroactive biomarkers. Suitable western blotting methods are described below in the examples section. For more rapid analysis (as may be important in emergency medical situations), immunosorbent assays (e.g., ELISA and RIA) and immunoprecipitation assays may be used. As one example, the biological sample or a portion thereof is immobilized on a substrate, such as a membrane made of nitrocellulose or PVDF; or a rigid substrate made of polystyrene or other plastic polymer such as a microtiter plate, and the substrate is contacted with an antibody that specifically binds GFAP, or one of the other neuroactive biomarkers under conditions that allow binding of antibody to the biomarker being analyzed. After washing, the presence of the antibody on the substrate indicates that the sample contained the marker being assessed. If the antibody is directly conjugated with a detectable label, such as an enzyme, fluorophore, or radioisotope, the presence of the label is optionally detected by examining the substrate for the detectable label. Alternatively, a detectably labeled secondary antibody that binds the marker-specific antibody is added to the substrate. The presence of detectable label on the substrate after washing indicates that the sample contained the marker.

[0041] Numerous permutations of these basic immunoassays are also operative in the invention. These include the biomarker-specific antibody, as opposed to the sample being immobilized on a substrate, and the substrate is contacted with GFAP or another neuroactive biomarker conjugated with a detectable label under conditions that cause binding of antibody to the labeled marker. The substrate is then contacted with a sample under conditions that allow binding of the marker being analyzed to the antibody. A reduction in the amount of detectable label on the substrate after washing indicates that the sample contained the marker.

[0042] Although antibodies are preferred for use in the invention because of their extensive characterization, any other suitable agent (e.g., a peptide, an aptamer, or a small organic molecule) that specifically binds GFAP or another neuroactive biomarker is optionally used in place of the antibody in the above described immunoassays. For example, an aptamer that specifically binds all spectrin and/or one or more of its SBDPs might be used. Aptamers are nucleic acid-based molecules that bind specific ligands. Methods for making aptamers with a particular binding specificity are known as detailed in U.S. Patent Nos. 5,475,096; 5,670,637; 5,696,249; 5,270,163; 5,707,796; 5,595,877; 5,660,985; 5,567,588; 5,683,867; 5,637,459; and 6,011,020.

[0043] A myriad of detectable labels that are operative in a diagnostic assay for biomarker expression are known in the art. Agents used in methods for detecting GFAP or another neuroactive biomarker are conjugated to a detectable label, e.g., an enzyme such as horseradish peroxidase. Agents labeled with horseradish peroxidase can be detected by adding an appropriate substrate that produces a color change in the presence of horseradish peroxidase. Several other detectable labels that may be used are known. Common examples of these include alkaline phosphatase, horseradish peroxidase, fluorescent compounds, luminescent compounds, colloidal gold, magnetic particles, biotin, radioisotopes, and other enzymes. It is appreciated that a primary/secondary antibody system is optionally used to detect one

or more biomarkers. A primary antibody that specifically recognizes one or more biomarkers is exposed to a biological sample that may contain the biomarker of interest. A secondary antibody with an appropriate label that recognizes the species or isotype of the primary antibody is then contacted with the sample such that specific detection of the one or more biomarkers in the sample is achieved.

5 [0044] The present invention employs a step of correlating the presence or amount of GFAP alone, or with one or more other neuroactive biomarker in a biological sample with the severity and/or type of nerve cell injury. GFAP measurement alone is shown herein to be highly effective in detecting MMTBI. The amount of GFAP and one or more other neuroactive biomarkers in the biological sample are associated with a neurological condition such as traumatic brain injury as detailed in the examples. The results of an inventive assay to synergistically measure GFAP and one or more
10 other neuroactive biomarkers can help a physician or veterinarian determine the type and severity of injury with implications as to the types of cells that have been compromised. These results are in agreement with CT scan and GCS results, yet are quantitative, obtained more rapidly, and at far lower cost.

[0045] The present invention provides a step of comparing the quantity of GFAP and the amount of at least one other neuroactive biomarker to normal levels to determine the neurological condition of the subject. It is appreciated that
15 selection of additional biomarkers allows one to identify the types of cells implicated in an abnormal neurological condition as well as the nature of cell death in the case of an axonal injury marker, namely an SBDP. The practice of an inventive process provides a test which can help a physician determine suitable therapeutics to administer for optimal benefit of the subject. While the data provided in the examples herein are provided with respect to a full spectrum of traumatic brain injury, it is appreciated that these results are applicable to ischemic events, neurodegenerative disorders, prion
20 related disease, epilepsy, chemical etiology and peripheral nervous system pathologies. As is shown in the subsequently provided example data, a gender difference is unexpectedly noted in abnormal subject neurological condition.

[0046] An assay for analyzing cell damage in a subject or a cell culture isolated therefrom is also provided. The assay includes: (a) a substrate for holding a sample isolated from a subject suspected of having a damaged nerve cell, the
25 sample being a fluid in communication with the nervous system of the subject prior to being isolated from the subject; (b) a GFAP specific binding agent; (c) a binding agent specific for another neuroactive biomarker, wherein said another neuroactive biomarker is UCH-L1 and (d) printed instructions for performing the assay illustratively for reacting: the specific binding agent with the biological sample or a portion of the biological sample to detect the presence or amount
30 of biomarker, and the agent specific for another neuroactive biomarker with the biological sample or a portion of the biological sample to detect the presence or amount of the at least one biomarker in the biological sample. The inventive assay can be used to detect a neurological condition for financial remuneration.

[0047] The assay optionally includes a detectable label such as one conjugated to the agent, or one conjugated to a substance that specifically binds to the agent, such as a secondary antibody.

[0048] An inventive process illustratively includes diagnosing a neurological condition in a subject, wherein said neurological condition is selected from traumatic brain injury and stroke. The biological sample is assayed by mechanisms
35 known in the art for detecting or identifying the presence of one or more biomarkers present in the biological sample. Based on the amount or presence of a target biomarker in a biological sample, a ratio of one or more biomarkers is optionally calculated. The ratio is optionally the level of one or more biomarkers relative to the level of another biomarker in the same or a parallel sample, or the ratio of the quantity of the biomarker to a measured or previously established baseline level of the same biomarker in a subject known to be free of a pathological neurological condition. The ratio
40 allows for the diagnosis of a neurological condition in the subject. An inventive process also optionally administers a therapeutic to the subject that will either directly or indirectly alter the ratio of one or more biomarkers.

[0049] A process is also described for diagnosing and optionally treating a multiple-organ injury. Multiple organs illustratively include subsets of neurological tissue such as brain, spinal cord and the like, or specific regions of the brain such as cortex, hippocampus and the like. Multiple injuries illustratively include apoptotic cell death which is detectable
45 by the presence of caspase induced SBDPs, and oncotic cell death which is detectable by the presence of calpain induced SBDPs. The inventive process illustratively includes assaying for a plurality of biomarkers in a biological sample obtained from a subject wherein the biological was optionally in fluidic contact with an organ suspected of having undergone injury or control organ when the biological sample was obtained from the subject. The inventive process determines a first subtype of organ injury based on a first ratio of a plurality of biomarkers. The inventive process also
50 determines a second subtype of a second organ injury based on a second ratio of the plurality of biomarkers in the biological sample. The ratios are illustratively determined by processes described herein or known in the art.

[0050] The subject invention illustratively includes a composition for distinguishing the magnitude of a neurological condition in a subject. An inventive composition is either an agent entity or a mixture of multiple agents. In a preferred embodiment a composition is a mixture. The mixture optionally contains a biological sample derived from a subject. The
55 subject is optionally suspected of having a neurological condition. The biological sample in communication with the nervous system of the subject prior to being isolated from the subject. In inventive composition also contains at least two primary agents, preferably antibodies, that specifically and independently bind to at least two biomarkers that may be present in the biological sample. In a preferred embodiment the first primary agent is in antibody that specifically

binds GFAP. A second primary agent is preferably an antibody that specifically binds a ubiquitin carboxyl-terminal hydrolase, preferably UCH-L1, or a spectrin breakdown product.

[0051] The agents of the inventive composition are optionally immobilized or otherwise in contact with a substrate. The inventive teachings are also preferably labeled with at least one detectable label. In a preferred embodiment the detectable label on each agent is unique and independently detectable in either the same assay chamber or alternate chambers. Optionally a secondary agent specific for detecting or binding to the primary agent is labeled with at least one detectable label. In the nonlimiting example the primary agent is a rabbit derived antibody. A secondary agent is optionally an antibody specific for a rabbit derived primary antibody. Mechanisms of detecting antibody binding to an antigen are well known in the art, and a person of ordinary skill in the art readily envisions numerous methods and agents suitable for detecting antigens or biomarkers in a biological sample.

[0052] The invention employs a step of correlating the presence or amount of a biomarker in a biological sample with the severity and/or type of nerve cell (or other biomarker-expressing cell) injury. The amount of biomarker(s) in the biological sample directly relates to severity of nerve tissue injury as a more severe injury damages a greater number of nerve cells which in turn causes a larger amount of biomarker(s) to accumulate in the biological sample (e.g., CSF; serum). Whether a nerve cell injury triggers an apoptotic and/or necrotic type of cell death can also be determined by examining the SBDPs present in the biological sample. Necrotic cell death preferentially activates calpain, whereas apoptotic cell death preferentially activates caspase-3. Because calpain and caspase-3 SBDPs can be distinguished, measurement of these markers indicates the type of cell damage in the subject. For example, necrosis-induced calpain activation results in the production of SBDP150 and SBDP145; apoptosis-induced caspase-3 activation results in the production of SBDP150i and SBDP120; and activation of both pathways results in the production of all four markers. Also, the level of or kinetic extent of UCH-L1 present in a biological sample may optionally distinguish mild injury from a more severe injury. In an illustrative example, severe MCAO (2h) produces increased UCH-L1 in both CSF and serum relative to mild challenge (30 min) while both produce UCH-L1 levels in excess of uninjured subjects. Moreover, the persistence or kinetic extent of the markers in a biological sample is indicative of the severity of the injury with greater injury indicating increases persistence of GFAP, UCH-L1, or SBDP in the subject that is measured by an inventive process in biological samples taken at several time points following injury.

[0053] The results of such a test can help a physician determine whether the administration a particular therapeutic such as calpain and/or caspase inhibitors or muscarinic cholinergic receptor antagonists might be of benefit to a patient. This method may be especially important in detecting age and gender difference in cell death mechanism.

[0054] It is appreciated that other reagents such as assay grade water, buffering agents, membranes, assay plates, secondary antibodies, salts, and other ancillary reagents are available from vendors known to those of skill in the art. Illustratively, assay plates are available from Corning, Inc. (Corning, NY) and reagents are available from Sigma-Aldrich Co. (St. Louis, MO).

[0055] Methods involving conventional biological techniques are described herein. Such techniques are generally known in the art and are described in detail in methodology treatises such as *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989; and *Current Protocols in Molecular Biology*, ed. Ausubel et al., Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates). Immunological methods (e.g., preparation of antigen-specific antibodies, immunoprecipitation, and immunoblotting) are described, e.g., in *Current Protocols in Immunology*, ed. Coligan et al., John Wiley & Sons, New York, 1991; and *Methods of Immunological Analysis*, ed. Masseyeff et al., John Wiley & Sons, New York, 1992.

[0056] Various aspects of the present invention are illustrated by the following non-limiting examples. The examples are for illustrative purposes and are not a limitation on any practice of the present invention. It will be understood that variations and modifications can be made without departing from the spirit and scope of the invention. While the examples are generally directed to mammalian tissue, specifically, analyses of mouse tissue, a person having ordinary skill in the art recognizes that similar techniques and other techniques known in the art readily translate the examples to other mammals such as humans. Reagents illustrated herein are commonly cross reactive between mammalian species or alternative reagents with similar properties are commercially available, and a person of ordinary skill in the art readily understands where such reagents may be obtained. Variations within the concepts of the invention are apparent to those skilled in the art.

Example 1: Materials for Biomarker Analyses.

[0057] Illustrative reagents used in performing the subject invention include Sodium bicarbonate (Sigma Cat #: C-3041), blocking buffer (Startingblock T20-TBS) (Pierce Cat#: 37543), Tris buffered saline with Tween 20 (TBST; Sigma Cat #: T-9039). Phosphate buffered saline (PBS; Sigma Cat #: P-3813); Tween 20 (Sigma Cat #: P5927); Ultra TMB ELISA (Pierce Cat #: 34028); and Nunc maxisorp ELISA plates (Fisher). Monoclonal and polyclonal GFAP and UCH-L1 antibodies are made in-house or are obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Antibodies directed to α -II spectrin and breakdown products as well as to MAP2 are available from Santa Cruz Biotechnology, Santa Cruz,

CA. Labels for antibodies of numerous subtypes are available from Invitrogen, Corp., Carlsbad, CA. Protein concentrations in biological samples are determined using bicinchoninic acid microprotein assays (Pierce Inc., Rockford, IL, USA) with albumin standards. All other necessary reagents and materials are known to those of skill in the art and are readily ascertainable.

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Example 2: Biomarker Assay Development

[0058] Anti-biomarker specific rabbit polyclonal antibody and monoclonal antibodies are produced in the laboratory. To determine reactivity specificity of the antibodies to detect a target biomarker a known quantity of isolated or partially isolated biomarker is analyzed or a tissue panel is probed by western blot. An indirect ELISA is used with the recombinant biomarker protein attached to the ELISA plate to determine optimal concentration of the antibodies used in the assay. Microplate wells are coated with rabbit polyclonal anti-human biomarker antibody. After determining the concentration of rabbit anti-human biomarker antibody for a maximum signal, the lower detection limit of the indirect ELISA for each antibody is determined. An appropriate diluted sample is incubated with a rabbit polyclonal antihuman biomarker antibody for 2 hours and then washed. Biotin labeled monoclonal anti-human biomarker antibody is then added and incubated with captured biomarker. After thorough wash, streptavidin horseradish peroxidase conjugate is added. After 1 hour incubation and the last washing step, the remaining conjugate is allowed to react with substrate of hydrogen peroxide tetramethyl benzadine. The reaction is stopped by addition of the acidic solution and absorbance of the resulting yellow reaction product is measured at 450 nanometers. The absorbance is proportional to the concentration of the biomarker. A standard curve is constructed by plotting absorbance values as a function of biomarker concentration using calibrator samples and concentrations of unknown samples are determined using the standard curve.

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Example 3: *In vivo* model of TBI injury model:

[0059] A controlled cortical impact (CCI) device is used to model TBI on rats as previously described (Pike et al, 1998). Adult male (280-300 g) Sprague-Dawley rats (Harlan: Indianapolis, IN) are anesthetized with 4% isoflurane in a carrier gas of 1:1 O₂/N₂O (4 min.) and maintained in 2.5% isoflurane in the same carrier gas. Core body temperature is monitored continuously by a rectal thermistor probe and maintained at 37 ± 1°C by placing an adjustable temperature controlled heating pad beneath the rats. Animals are mounted in a stereotactic frame in a prone position and secured by ear and incisor bars. Following a midline cranial incision and reflection of the soft tissues, a unilateral (ipsilateral to site of impact) craniotomy (7 mm diameter) is performed adjacent to the central suture, midway between bregma and lambda. The dura mater is kept intact over the cortex. Brain trauma is produced by impacting the right (ipsilateral) cortex with a 5 mm diameter aluminum impactor tip (housed in a pneumatic cylinder) at a velocity of 3.5 m/s with a 1.6 mm compression and 150 ms dwell time. Sham-injured control animals are subjected to identical surgical procedures but do not receive the impact injury. Appropriate pre-and post-injury management is preformed to insure compliance with guidelines set forth by the University of Florida Institutional Animal Care and Use Committee and the National Institutes of Health guidelines detailed in the Guide for the Care and Use of Laboratory Animals. In addition, research is conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the "Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition."

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Example 4: Middle cerebral artery occlusion (MCAO) injury model:

[0060] Rats are incubated under isoflurane anesthesia (5% isoflurane *via* induction chamber followed by 2% isoflurane *via* nose cone), the right common carotid artery (CCA) of the rat is exposed at the external and internal carotid artery (ECA and ICA) bifurcation level with a midline neck incision. The ICA is followed rostrally to the pterygopalatine branch and the ECA is ligated and cut at its lingual and maxillary branches. A 3-0 nylon suture is then introduced into the ICA *via* an incision on the ECA stump (the suture's path was visually monitored through the vessel wall) and advanced through the carotid canal approximately 20 mm from the carotid bifurcation until it becomes lodged in the narrowing of the anterior cerebral artery blocking the origin of the middle cerebral artery. The skin incision is then closed and the endovascular suture left in place for 30 minutes or 2 hours. Afterwards the rat is briefly reanesthetized and the suture filament is retracted to allow reperfusion. For sham MCAO surgeries, the same procedure is followed, but the filament is advanced only 10 mm beyond the internal-external carotid bifurcation and is left in place until the rat is sacrificed. During all surgical procedures, animals are maintained at 37 ± 1°C by a homeothermic heating blanket (Harvard Apparatus, Holliston, MA, U.S.A.). It is important to note that at the conclusion of each experiment, if the rat brains show pathologic evidence of subarachnoid hemorrhage upon necropsy they are excluded from the study. Appropriate pre-and post-injury management is preformed to insure compliance with all animal care and use guidelines.

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Example 5: Tissue and Sample Preparation:

[0061] At the appropriate time points (2, 6, 24 hours and 2, 3, 5 days) after injury, animals are anesthetized and immediately sacrificed by decapitation. Brains are quickly removed, rinsed with ice cold PBS and halved. The right hemisphere (cerebrocortex around the impact area and hippocampus) is rapidly dissected, rinsed in ice cold PBS, snap-frozen in liquid nitrogen, and stored at -80°C until used. For immunohistochemistry, brains are quick frozen in dry ice slurry, sectioned via cryostat (20 μm) onto SUPERFROST PLUS GOLD® (Fisher Scientific) slides, and then stored at -80°C until used. For the left hemisphere, the same tissue as the right side is collected. For Western blot analysis, the brain samples are pulverized with a small mortar and pestle set over dry ice to a fine powder. The pulverized brain tissue powder is then lysed for 90 min at 4°C in a buffer of 50 mM Tris (pH 7.4), 5 mM EDTA, 1% (v/v) Triton X-100, 1 mM DTT, 1x protease inhibitor cocktail (Roche Biochemicals). The brain lysates are then centrifuged at 15,000xg for 5 min at 4°C to clear and remove insoluble debris, snap-frozen, and stored at -80°C until used.

[0062] For gel electrophoresis and electroblotting, cleared CSF samples (7 μl) are prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 2X loading buffer containing 0.25 M Tris (pH 6.8), 0.2 M DTT, 8% SDS, 0.02% bromophenol blue, and 20% glycerol in distilled H₂O. Twenty micrograms (20 μg) of protein per lane are routinely resolved by SDS-PAGE on 10-20% Tris/glycine gels (Invitrogen, Cat #EC61352) at 130 V for 2 hours. Following electrophoresis, separated proteins are laterally transferred to polyvinylidene fluoride (PVDF) membranes in a transfer buffer containing 39 mM glycine, 48 mM Tris-HCl (pH 8.3), and 5% methanol at a constant voltage of 20 V for 2 hours at ambient temperature in a semi-dry transfer unit (Bio-Rad). After electro-transfer, the membranes are blocked for 1 hour at ambient temperature in 5% non-fat milk in TBS and 0.05% Tween-2 (TBST) then are incubated with the primary polyclonal UCH-L1 antibody in TBST with 5% non-fat milk at 1:2000 dilution as recommended by the manufacturer at 4°C overnight. This is followed by three washes with TBST, a 2 hour incubation at ambient temperature with a biotinylated linked secondary antibody (Amersham, Cat # RPN1177v1), and a 30 min incubation with Streptavidin-conjugated alkaline phosphatase (BCIP/NBT reagent: KPL, Cat # 50-81-08). Molecular weights of intact biomarker proteins are assessed using rainbow colored molecular weight standards (Amersham, Cat # RPN800V). Semi-quantitative evaluation of intact GFAP, UCH-L1, or SBDP protein levels is performed via computer-assisted densitometric scanning (Epson XL3500 scanner) and image analysis with ImageJ software (NIH).

Example 6: UCH-L1 is increased in CSF following MCAO challenge.

[0063] Subjects are subjected to MCAO challenge and CSF samples analyzed by quantitative western blotting. UCH-L1 protein is readily detectable after injury at statically significant levels above the amounts of UCH-L1 in sham treated samples (FIGs. 1A, B). These UCH-L1 levels are transiently elevated (at 6 h) after mild ischemia (30 min MCAO) followed by reperfusion, while levels are sustained from 6 to 72 h after a more severe (2 h MCAO) ischemia (FIGs. 1A, B).

Example 7: ELISA readily identifies UCH-L1 levels in both CSF and serum.

[0064] ELISA is used to more rapidly and readily detect and quantitate UCH-L1 in biological samples. For a UCH-L1 sandwich ELISA (swELISA), 96-well plates are coated with 100 μl/well capture antibody (500 ng/well purified rabbit anti-UCH-L1, made in-house by conventional techniques) in 0.1 M sodium bicarbonate, pH 9.2. Plates are incubated overnight at 4°C, emptied and 300 μl/well blocking buffer (Startingblock T20-TBS) is added and incubated for 30 min at ambient temperature with gentle shaking. This is followed by either the addition of the antigen standard (recombinant UCH-L1) for standard curve (0.05 - 50 ng/well) or samples (3-10 μl CSF) in sample diluent (total volume 100 μl/well). The plate is incubated for 2 hours at room temperature, then washed with automatic plate washer (5 x 300 μl/well with wash buffer, TBST). Detection antibody mouse anti-UCH-L1-HRP conjugated (made in-house, 50 μg/ml) in blocking buffer is then added to wells at 100 μl/well and incubated for 1.5 h at room temperature, followed by washing. If amplification is needed, biotinyl-tyramide solution (Perkin Elmer Elast Amplification Kit) is added for 15 min at room temperature, washed then followed by 100 μl/well streptavidin-HRP (1:500) in PBS with 0.02% Tween-20 and 1% BSA for 30 min and then followed by washing. Lastly, the wells are developed with 100 μl/well TMB substrate solution (Ultra-TMB ELISA, Pierce# 34028) with incubation times of 5-30 minutes. The signal is read at 652 nm with a 96-well spectrophotometer (Molecular Device Spectramax 190).

[0065] UCH-L1 levels of the TBI group (percussive injury) are significantly higher than the sham controls (p<0.01, ANOVA analysis) and the naive controls as measured by a swELISA demonstrating that UCH-L1 is elevated early in CSF (2h after injury) then declines at around 24 h after injury before rising again 48 h after injury (FIG. 2).

[0066] Following MCAO challenge the magnitude of UCH-L1 in CSF is dramatically increased with severe (2h) challenge relative to a more mild challenge (30 min). (FIG. 3) The more severe 2 h MCAO group UCH-L1 protein levels are 2-5 fold higher than 30 min MCAO (p < 0.01, ANOVA analysis). UCH-L1 protein levels for shams are virtually indistinguishable from naive controls.

[0067] Similar results are obtained for UCH-L1 in serum. Blood (3-4 ml) is collected at the end of each experimental period directly from the heart using syringe equipped with 21 gage needle placed in a polypropylene tube and allowed to stand for 45 min to 1 hour at room temperature to form clot. Tubes are centrifuged for 20 min at 3,000xg and the serum removed and analyzed by ELISA (FIGS. 4, 5).

[0068] UCH-L1 levels of the TBI group are significantly higher than the sham group ($p < 0.001$, ANOVA analysis) and for each time point tested 2 h through 24 h respective to the same sham time points ($p < 0.005$, Student's T-test). UCH-L1 is significantly elevated after injury as early as 2h in serum. Severe MCAO challenge produces increased serum UCH-L1 relative to mild challenge. Both mild and severe challenge are statistically higher than sham treated animals indicating that serum detection of UCH-L1 is a robust diagnostic and the levels are able to sufficiently distinguish mild from severe injury.

Example 8: Analysis of spectrin breakdown products:

[0069] Spectrin breakdown products are analyzed following rat MCAO challenge by procedures similar to those described in U.S. Patent No. 7,291,710, the contents of which are incorporated herein by reference. FIG. 6 demonstrates that levels of SBDP145 in both serum and CSF are significantly ($p < 0.05$) increased at all time points studied following severe (2hr) MCAO challenge relative to mild (30 min) challenge. Similarly, SBDP120 demonstrates significant elevations following severe MCAO challenge between 24 and 72 hours after injury in CSF (FIG. 7). However, levels of SBDP120 in serum are increased following severe challenge relative to mild challenge at all time points between 2 and 120 hours. In both CSF and Serum both mild and severe MCAO challenge produces increased SPBP120 and 140 relative to sham treated subjects.

Example 9: Analysis of MAP2:

[0070] Microtubule Associated Protein 2 (MAP2) is assayed as a biomarker in both CSF and serum following mild (30 min) and severe (2 hr) MCAO challenge in subjects by ELISA or western blotting essentially as described herein. Antibodies to MAP2 (MAP-2 (E-12)) are obtained from Santa Cruz Biotechnology, Santa Cruz, CA. These antibodies are suitable for both ELISA and western blotting procedures and are crossreactive to murine and human MAP2. Levels of MAP2 are significantly ($p < 0.05$) increased in subjects following mild MCAO challenge relative to naive animals in both CSF and serum (FIG. 8). Similar to UCH-L1 and SBDPs, severe challenge (2 hr) produces much higher levels of MAP2 in both samples than mild challenge (30 min).

Example 10: Severe Traumatic Brain Injury Study

[0071] A study was conducted that included 46 human subjects suffering severe traumatic brain injury. Each of these subjects is characterized by being over age 18, having a GCS of less than or equal to 8 and required ventriculostomy and neuromonitoring as part of routine care. A control group A, synonymously detailed as CSF controls, included 10 individuals also being over the age of 18 or older and no injuries. Samples are obtained during spinal anesthesia for routine surgical procedures or access to CSF associated with treatment of hydrocephalus or meningitis. A control group B, synonymously described as normal controls, totaled 64 individuals, each age 18 or older and experiencing multiple injuries without brain injury. Further details with respect to the demographics of the study are provided in Table 1.

Table 1. Subject Demographics for Severe Traumatic Brain Injury Study

		TBI	CSF Controls	Normal Controls
Number		46	10	64
	Males	34 (73.9%)	29 (65.9%)	26 (40.6%)
	Females	12 (26.1%)	15 (34.1%)	38 (59.4%)
Age:	Average	50.2	58.2 1,2	30.09 2,3
	Std Dev	19.54	20.52	15.42
	Minimum	19	23	18
	Maximum	88	82	74
Race:	Caucasian			
	Black	45	38 (86.4%)	52 (81.2%)
	Asian	1	6 (13.6)	4 (6.3%)
	Other			7 (10.9%)

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(continued)

	TBI	CSF Controls	Normal Controls
			1 (1.6%)
GCS in Emergency Department			
	Average	5.3	
	Std Dev	1.9	

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10 **[0072]** The level of biomarkers found in the first available CSF and serum samples obtained in the study are provided in FIGs. 9 and 10, respectively. The average first CSF sample collected as detailed in FIG. 9 was 11.2 hours while the average time for collection of a serum sample subsequent to injury event as per FIG. 10 is 10.1 hours. The quantity of each of the biomarkers of UCH-L1, MAP2, SBDP145, SBDP120, and GFAP are provided for each sample for the cohort of traumatic brain injury sufferers as compared to a control group. The diagnostic utility of the various biomarkers within 15 the first 12 hours subsequent to injury based on a compilation of CSF and serum data is provided in Fig. 11 and indicates in particular the value of GFAP as well as that of additional markers UCH-L1 and the spectrin breakdown products. Elevated levels of UCH-L1 are indicative of the compromise of neuronal cell body damage while an increase in SPDP145 with a corresponding decrease in SBDP120 is suggestive of acute axonal necrosis.

20 **[0073]** One subject from the traumatic brain injury cohort was a 52 year old Caucasian woman who had been involved in a motorcycle accident while not wearing a helmet. Upon admission to an emergency room her GCS was 3 and during the first 24 hours subsequent to trauma her best GCS was 8. After 10 days her GCS was 11. CT scanning revealed SAH and facial fractures with a Marshall score of 11 and a Rotterdam score of 2. Ventriculostomy was removed after 5 years and an overall good outcome was obtained. Arterial blood pressure (MABP), intracranial pressure (ICP) and cerebral perfusion pressure (CPP) for this sufferer of traumatic brain injury as a function of time is depicted in FIG. 12. 25 A possible secondary insult is noted at approximately 40 hours subsequent to the injury as noted by a drop in MABP and CPP. The changes in concentration of inventive biomarkers per CSF and serum samples from this individual are noted in FIG. 13. These results include a sharp increase in GFAP in both the CSF and serum as well as the changes in the other biomarkers depicted in FIG. 13 and provide important clinical information as to the nature of the injury and the types of cells involved, as well as modes of cell death associated with the spectrin breakdown products.

30 **[0074]** Another individual of the severe traumatic brain injury cohort included a 51 year old Caucasian woman who suffered a crush injury associated with a horse falling on the individual. GCS on admission to emergency room was 3 with imaging analysis initially being unremarkable with minor cortical and subcortical contusions. MRI on day 5 revealed significant contusions in posterior fossa. The Marshall scale at that point was indicated to be 11 with a Rotterdam scale score of 3. The subject deteriorated and care was withdrawn 10 days after injury. The CSF and serum values for this individual during a period of time are provided in FIG. 14. 35

[0075] Based on the sandwich ELISA testing, GFAP values as a function of time are noted to be markedly elevated relative to normal controls (control group B) as a function of time.

40 **[0076]** The concentration of spectrin breakdown products, MAP2 and UCH-L1 as a function of time subsequent to traumatic brain injury has been reported elsewhere as exemplified in U.S. Patents 7,291,710 and 7,396,654 each of which is incorporated herein by reference.

45 **[0077]** An analysis was performed to evaluate the ability of biomarkers measured in serum to predict TBI outcome, specifically GCS. Stepwise regression analysis was the statistical method used to evaluate each of the biomarkers as an independent predictive factor, along with the demographic factors of age and gender, and also interactions between pairs of factors. Interactions determine important predictive potential between related factors, such as when the relationship between a biomarker and outcome may be different for men and women, such a relationship would be defined as a gender by biomarker interaction.

[0078] The resulting analysis identified biomarkers UCH-L1, MAP2, and GFAP as being statistically significant predictors of GCS (Table 2, 3). Furthermore, GFAP was shown to have improved predictability when evaluated in interaction with UCH-L1 and gender (Table 4, 5). 50

Table 2. Stepwise Regression Analysis 1 - Cohort includes: All Subjects >= 18 Years Old
Summary of Stepwise Selection - 48 Subjects

Variable	Parameter	Model			
Step Entered	Estimate	R-Square	F Value	p-value	
Intercept	13.02579				
2 SEXCD	-2.99242	0.1580	7.29	0.0098	
1 CSF_UCH_L1	-0.01164	0.2519	11.54	0.0015	

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(continued)

Summary of Stepwise Selection - 48 Subjects

Variable	Parameter	Model			
Step Entered	Estimate	R-Square	F Value	p-value	
3 Serum_MAP_2	0.96055	0.3226	4.59	0.0377	

Table 3. Stepwise Regression Analysis 2 - Cohort includes: TBI Subjects >= 18 Years Old

Summary of Stepwise Selection - 39 Subjects

Variable	Parameter	Model			
Step Entered	Estimate	R-Square	F Value	p-value	
Intercept	5.73685				
1 Serum_UCH_L1	-0.30025	0.0821	8.82	0.0053	
2 Serum_GFAP	0.12083	0.1973	5.16	0.0291	

Table 4. Stepwise Regression Analysis 1 - Cohort includes: TBI and A Subjects >= 18 Years Old

Summary of Stepwise Selection - 57 Subjects

Variable	Parameter	Model			
Step Entered	Estimate	R-Square	F Value	p-value	
Intercept	8.04382				
1 Serum_UCH_L	-0.92556	0.1126	12.90	0.0007	
2 Serum_MAP_2	1.07573	0.2061	5.79	0.0197	
3 Serum_UCH-L1 +Serum_GFAP	0.01643	0.2663	4.35	0.0419	

Table 5. Stepwise Regression Analysis 2 - Cohort includes: TBI Subjects >= 18 Years Old

Summary of Stepwise Selection - 44 Subjects

Variable	Parameter	Model			
Step Entered	Estimate	R-Square	F Value	p-value	
Intercept	5.50479				
1 Serum_UCH_L1	-0.36311	0.0737	11.95	0.0013	
2 SEX_Serum_GFAP	0.05922	0.1840	5.09	0.0296	
3 Serum_MAP_2	0.63072	0.2336	2.59	0.1157	

Example 11:

[0079] The study of Example 10 was repeated with a moderate traumatic brain injury cohort characterized by GCS scores of between 9 and 11, as well as a mild traumatic brain injury cohort characterized by GCS scores of 12-15. Blood samples were obtained from each patient on arrival to the emergency department of a hospital within 2 hours of injury and measured by ELISA for levels of GFAP in nanograms per milliliter. The results were compared to those of a control group who had not experienced any form of injury. Secondary outcomes included the presence of intracranial lesions in head CT scans.

[0080] Over 3 months 53 patients were enrolled: 35 with GCS 13-15, 4 with GCS 9-12 and 14 controls. The mean age was 37 years (range 18-69) and 66% were male. The mean GFAP serum level was 0 in control patients, 0.107 (0.012) in patients with GCS 13-15 and 0.366 (0.126) in GCS 9-12 (P<0.001). The difference between GCS 13-15 and controls was significant at P<0.001. In patients with intracranial lesions on CT GFAP levels were 0.234 (0.055) compared to 0.085 (0.003) in patients without lesions (P<0.001). There is a significant increase in GFAP in serum following a MTBI compared to uninjured controls in both the mild and moderate groups. GFAP was also significantly associated with the presence of intracranial lesions on CT.

[0081] FIG. 16 shows GFAP concentration for controls as well as individuals in the mild/moderate traumatic brain injury cohort as a function of CT scan results upon admission and 24 hours thereafter. Simultaneous assays were performed in the course of this study for UCH-L1 biomarker. The UCH-L1 concentration derived from the same samples

as those used to determine GFAP is provided FIG. 17. The concentration of UCH-L1 and GFAP as well as a biomarker not selected for diagnosis of neurological condition, S100b, is provided as a function of injury magnitude between control, mild, and moderate traumatic brain injury as shown in FIG. 18. The simultaneous analyses of UCH-L1 and GFAP from these patients illustrates the synergistic effect of the inventive process in allowing an investigator to simultaneously diagnose traumatic brain injury as well as discern the level of traumatic brain injury between mild and moderate levels of severity. FIG. 19 shows the concentration of the same markers as depicted in FIG. 18 with respect to initial evidence upon hospital admission as to lesions in tomography scans illustrating the high confidence in predictive outcome of the inventive process. FIG. 20 shows that both NSE and MAP2 are elevated in subjects with MTBI in serum both at admission and at 24 hours of follow up. These data demonstrate a synergistic diagnostic effect of measuring multiple biomarkers such as GFAP, UCH-L1, NSE, and MAP2 in a subject.

[0082] Through the simultaneous measurement of multiple biomarkers such as UCH-L1, GFAP, NSE, and MAP2, rapid and quantifiable determination as to the severity of the brain injury is obtained consistent with GSC scoring and CT scanning yet in a surprisingly more quantifiable, expeditious and economic process. Additionally, with a coupled assay for biomarkers indicative of neurological condition, the nature of the neurological abnormality is assessed and in this particular study suggestive of neuronal cell body damage. As with severe traumatic brain injury, gender variations are noted suggesting a role for hormonal anti-inflammatories as therapeutic candidates. A receiver operating characteristic (ROC) modeling of UCH-L1, GFAP and SBDP145 post TBI further supports the value of simultaneous measurement of these biomarkers, as shown in Figs. 21, 22.

[0083] In addition, Figure 22 showed that several brain biomarkers (GFAP, UCH-L1 and MAP2) in stroke patients' plasma. Samples were collected with an average post-injury time 24.2 hr (range 18-30 h). Top panel shows GFAP, UCH-L1 and MAP2 levels in stroke (n=11) versus normal controls (n=30). Bottom panel further shows that UCH-L1 is elevated with both hemorrhagic and ischemic stroke populations when compared to normal control plasma.

[0084] Patent documents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains.

[0085] The foregoing description is illustrative of particular embodiments of the invention, but is not meant to be a limitation upon the practice thereof. The following claims are intended to define the scope of the invention.

Claims

1. A process for determining the neurological condition of a subject wherein said neurological condition is selected from traumatic brain injury and stroke comprising:

measuring a biological sample obtained from the subject at a first time for a quantity of a first biomarker and a quantity of at least one additional neuroactive biomarker, wherein said first biomarker is GFAP and said at least one additional neuroactive biomarker is UCH-L1; and

comparing the quantity of said first biomarker and the quantity of said at least one additional neuroactive biomarker to the baseline level of said first biomarker and said at least one additional neuroactive biomarker in a subject known to be free of a pathological neurological condition to determine the neurological condition of the subject;

wherein said biological sample is cerebrospinal fluid (CSF), whole blood, serum, or plasma.

2. The process of claim 1, further comprising measuring for a quantity of a further neuroactive biomarker, wherein said further neuroactive biomarker is NSE, SBDP145, SBDP120, S100b, MAP2 or MBP.

3. The process of claim 1, further comprising measuring a second quantity of said first biomarker and a second quantity of said at least one additional neuroactive biomarker at a second time to yield a kinetic profile for said first biomarker and said at least one additional neuroactive biomarker.

4. The process of claim 1, further comprising comparing the quantity of said first biomarker and the quantity of said at least one additional neuroactive biomarker between the levels in the subject and the levels in other individuals of the same gender as the subject known to be free of a pathological neural condition.

5. The process of claim 4, wherein it is determined if the subject has been exposed to some degree of traumatic brain injury ranging from mild to severe; and wherein the process further comprises predicting mortality based on the quantity of UCH-L1 and the quantity of GFAP.

6. An assay product for determining the neurological condition, wherein said neurological condition is selected from

traumatic brain injury and stroke of a subject comprising:

5 a substrate for holding a biological sample isolated from the subject;
a first biomarker specifically binding agent wherein a first biomarker is GFAP;
an agent specifically binding at least one additional neuroactive biomarker wherein said at least one additional
neuroactive biomarker is UCH-L1; and
printed instructions for reacting said first biomarker specific agent with a first portion of said biological sample
so as to detect an amount of said first biomarker and reacting said at least one additional neuroactive biomarker
specific agent with a second portion of said biological sample and said at least one additional neuroactive
10 biomarker in said biological sample so as to detect an amount of said at least one additional neuroactive
biomarker to determine the neurological condition of the subject according to the process of claim 1.

7. The assay product of claim 6, wherein the first biomarker specific agent is an anti-GFAP antibody.
- 15 8. The assay product of claim 6, wherein said first biomarker specifically binding agent and said agent specifically
binding at least one additional neuroactive biomarker are both bound to the substrate, optionally with spatial overlap.
9. The assay product of any of claims 6 or 8, further comprising a separate first biomarker detection species for said
first biomarker and a separate discernable at least one additional neuroactive biomarker detection species for said
20 at least one additional neuroactive biomarker.
10. A process for determining if a subject has suffered mild traumatic brain injury or moderate traumatic brain injury in
an event comprising:

25 measuring by ELISA a biological sample obtained from said subject for a quantity of GFAP, wherein said
biological sample is whole blood, serum or plasma; and
comparing the measured quantity of said GFAP to normal levels of GFAP in a control group who had not
experienced any form of head injury, wherein if the measured quantity of GFAP is between 0.082 and 0.492
nanograms per milliliter, the subject has suffered mild traumatic brain injury or moderate traumatic brain injury
30 in the event.

Patentansprüche

- 35 1. Prozess zum Ermitteln des neurologischen Zustands eines Subjekts, wobei der neurologische Zustand ausgewählt
ist aus traumatischer Hirnverletzung und Schlaganfall umfassend:

40 messen einer biologischen Probe erhalten von dem Subjekt zum ersten Mal für ein Menge eines ersten Bio-
markers und für eine Menge mindestens eines zusätzlichen neuroaktiven Biomarkers, wobei der erste Biomarker
GFAP ist und der mindestens eine zusätzliche neuroaktive Biomarker UCH-L1 ist; und
vergleichen der Menge des ersten Biomarkers und der Menge des mindestens einen zusätzlichen neuroaktiven
Biomarkers mit dem Ausgangswert des ersten Biomarkers und des mindestens einen zusätzlichen neuroaktiven
Biomarker in einem Subjekt, von dem bekannt ist, dass es frei von einem pathologischen neurologischen
Zustand ist, um den neurologischen Zustand des Subjekts zu ermitteln;
45 wobei die biologische Probe Zerebrospinalflüssigkeit (CSF), Vollblut, Serum oder Plasma ist.

2. Prozess nach Anspruch 1, weiterhin umfassend messen einer Menge eines weiteren neuroaktiven Biomarkers,
wobei der weitere neuroaktive Biomarker NSE, SBDP145, SBDP120, S100b, MAP2 oder MBP ist.
- 50 3. Prozess nach Anspruch 1, weiterhin umfassend messen einer zweiten Menge des ersten Biomarkers und einer
zweiten Menge des mindestens einen zusätzlichen neuroaktiven Biomarkers zum zweiten Mal, um ein kinetisches
Profil für den ersten Biomarker und den mindestens einen zusätzlichen neuroaktiven Biomarker zu erbringen.
4. Prozess nach Anspruch 1, weiterhin umfassend vergleichen der Menge des ersten Biomarkers und der Menge des
mindestens einen zusätzlichen neuroaktiven Biomarkers zwischen den Werten in dem Subjekt und den Werten in
55 anderen Individuen des selben Geschlechts wie das Subjekt, von dem bekannt ist, dass es frei von einem patho-
logischen neuronalen Zustand ist.

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5. Prozess nach Anspruch 4, wobei es ermittelt wird, falls das Subjekt einem gewissen Grad an traumatischer Hirnverletzung angefangen von leicht bis schwer ausgesetzt wurde; und wobei der Prozess weiterhin umfasst vorher-sagen der Sterblichkeit basierend auf der Menge des UCH-L1 und der Menge des GFAP.

5 6. Assayprodukt zum Ermitteln des neurologischen Zustands, wobei der neurologische Zustand ausgewählt ist aus traumatischer Hirnverletzung und Schlaganfall eines Subjekts umfassend:

ein Substrat zum Halten einer von dem Subjekt isolierten biologischen Probe;
einen ersten Biomarker spezifisch bindenden Wirkstoff, wobei ein erster Biomarker GFAP ist;
10 ein Wirkstoff spezifisch mindestens einen zusätzlichen neuroaktiven Biomarker bindend, wobei der mindestens eine zusätzliche neuroaktive Biomarker UCH-L1 ist; und
ausgedruckte Anweisungen zum Reagieren des für den ersten Biomarker spezifischen Wirkstoffs mit einem ersten Teil der biologischen Probe, so dass eine Menge des ersten Biomarkers detektiert wird und zum Reagieren des für den mindestens einen zusätzlichen neuroaktiven Biomarker spezifischen Wirkstoffs mit einem zweiten
15 Teil der biologischen Probe und dem mindestens einen zusätzlichen neuroaktiven Biomarker in der biologischen Probe, so dass eine Menge des mindestens einen zusätzlichen neuroaktiven Biomarkers detektiert wird, um den neurologischen Zustand des Subjekts nach dem Prozess nach Anspruch 1 zu ermitteln.

20 7. Assayprodukt nach Anspruch 6, wobei der für den ersten Biomarker spezifische Wirkstoff ein anti-GFAP Antikörper ist.

8. Assayprodukt nach Anspruch 6, wobei der erste Biomarker spezifisch bindende Wirkstoff und der Wirkstoff spezifisch mindestens einen zusätzlichen neuroaktiven Biomarker bindend beide am Substrat gebunden sind, gegebenenfalls mit räumlicher Überlagerung.

25 9. Assayprodukt nach einem der Ansprüche 6 oder 8, weiterhin umfassend einen getrennten ersten Biomarker Speziesnachweis für den ersten Biomarker und einen getrennten erkennbaren mindestens einen zusätzlichen neuroaktiven Biomarker Speziesnachweis für den mindestens einen zusätzlichen neuroaktiven Biomarker.

30 10. Prozess zum Ermitteln, falls ein Subjekt an leichter traumatischer Hirnverletzung oder an mäßiger traumatischer Hirnverletzung in einem Fall gelitten hat umfassend:

messen einer von dem Subjekt erhaltenen biologischen Probe mit Hilfe von ELISA für eine Menge von GFAP, wobei die biologische Probe Vollblut, Serum oder Plasma ist; und vergleichen der gemessenen Menge an GFAP zu normalen Werten von GFAP in einer Kontrollgruppe, die keine Form von Kopfverletzung erfahren hatte, wobei falls die gemessene Menge an GFAP zwischen 0.082 und 0.492 Nanogramm pro Milliliter ist, hat das Subjekt leichte traumatische Hirnverletzung oder mäßige traumatische Hirnverletzung in dem Fall erlitten.

40 **Revendications**

1. Procédé pour déterminer l'état neurologique d'un sujet, dans lequel ledit état neurologique est sélectionné parmi un traumatisme cérébral et un accident vasculaire cérébral, comprenant :

45 la mesure d'un échantillon biologique obtenu à partir du sujet à un premier moment pour une quantité d'un premier biomarqueur et une quantité d'au moins un biomarqueur neuroactif supplémentaire, ledit premier biomarqueur étant GFAP et ledit au moins un biomarqueur neuroactif supplémentaire étant UCH-L1 ; et
la comparaison de la quantité dudit premier biomarqueur et de la quantité dudit au moins un biomarqueur neuroactif supplémentaire au niveau de base dudit premier biomarqueur et dudit au moins un biomarqueur
50 neuroactif supplémentaire chez un sujet que l'on sait dépourvu d'un état neurologique pathologique pour déterminer l'état neurologique du sujet ;
ledit échantillon biologique étant du liquide céphalorachidien (LCR), du sang total, du sérum ou du plasma.

55 2. Procédé de la revendication 1, comprenant en outre la mesure pour une quantité d'un autre biomarqueur neuroactif, ledit autre biomarqueur neuroactif étant NSE, SBDP145, SBDP120, S100b, MAP2 ou MBP.

3. Procédé de la revendication 1, comprenant en outre la mesure d'une seconde quantité dudit premier biomarqueur et d'une seconde quantité dudit au moins un biomarqueur neuroactif supplémentaire à un second moment pour

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fournir un profil cinétique pour ledit premier biomarqueur et ledit au moins un biomarqueur neuroactif supplémentaire.

5 4. Procédé de la revendication 1, comprenant en outre la comparaison de la quantité dudit premier biomarqueur et de la quantité dudit au moins un biomarqueur neuroactif supplémentaire entre les niveaux chez le sujet et les niveaux chez d'autres individus du même sexe que le sujet que l'on sait être dépourvus d'un état neurologique pathologique.

10 5. Procédé de la revendication 4, dans lequel on détermine si le sujet a été exposé à un certain degré de traumatisme cérébral allant de léger à grave ; et dans lequel le procédé comprend en outre la prédiction de la mortalité sur la base de la quantité d'UCH-L1 et de la quantité de GFAP.

6. Produit de test pour déterminer l'état neurologique, dans lequel ledit état neurologique est sélectionné parmi un traumatisme cérébral et un accident vasculaire cérébral d'un sujet, comprenant :

15 un substrat pour maintenir un échantillon biologique isolé à partir du sujet ;

un agent se liant spécifiquement au premier biomarqueur, un premier biomarqueur étant GFAP ;

un agent se liant spécifiquement à au moins un biomarqueur neuroactif supplémentaire, ledit au moins un biomarqueur neuroactif supplémentaire étant UCH-L1 ; et

20 des instructions imprimées pour faire réagir ledit agent spécifique au premier biomarqueur avec une première partie dudit échantillon biologique de façon à détecter une quantité dudit premier biomarqueur et faire réagir ledit agent spécifique au au moins un biomarqueur neuroactif supplémentaire avec une seconde partie dudit échantillon biologique et dudit au moins un biomarqueur neuroactif supplémentaire dans ledit échantillon biologique de façon à détecter une quantité dudit au moins un biomarqueur neuroactif supplémentaire pour déterminer l'état neurologique du sujet selon le procédé de la revendication 1.

25 7. Produit de test de la revendication 6, dans lequel l'agent spécifique au premier biomarqueur est un anticorps anti-GFAP.

30 8. Produit de test de la revendication 6, dans lequel ledit agent se liant spécifiquement au premier biomarqueur et ledit agent se liant spécifiquement à au moins un biomarqueur neuroactif supplémentaire sont tous les deux liés au substrat, facultativement avec un chevauchement spatial.

35 9. Produit de test de l'une quelconque des revendications 6 ou 8, comprenant en outre une espèce distincte de détection du premier biomarqueur pour ledit premier biomarqueur et une espèce distincte, discernable, de détection du au moins un biomarqueur neuroactif supplémentaire pour ledit au moins un biomarqueur neuroactif supplémentaire.

10. Procédé pour déterminer si un sujet a subi un traumatisme cérébral léger ou un traumatisme cérébral modéré lors d'un événement, comprenant :

40 la mesure par ELISA d'un échantillon biologique obtenu à partir dudit sujet pour une quantité de GFAP, ledit échantillon biologique étant du sang total, du sérum ou du plasma ; et

45 la comparaison de la quantité mesurée de ladite GFAP à des niveaux normaux de GFAP dans un groupe témoin qui n'avait subi aucune forme de traumatisme crânien, où, si la quantité mesurée de GFAP est entre 0,082 et 0,492 nanogramme par millilitre, le sujet a subi un traumatisme cérébral léger ou un traumatisme cérébral modéré lors de l'événement.

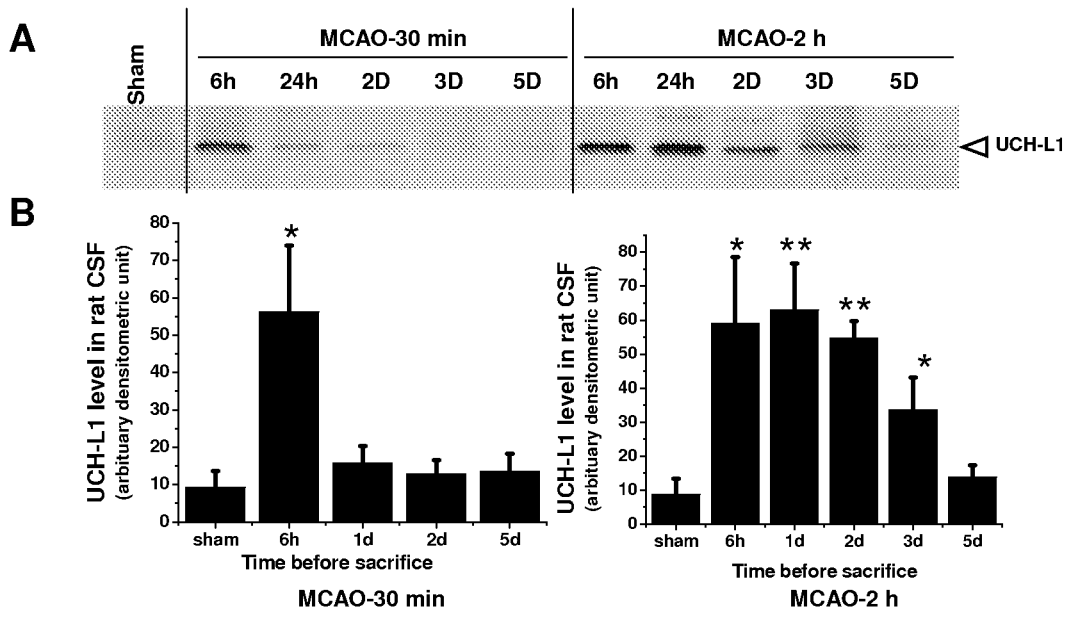


Figure 1

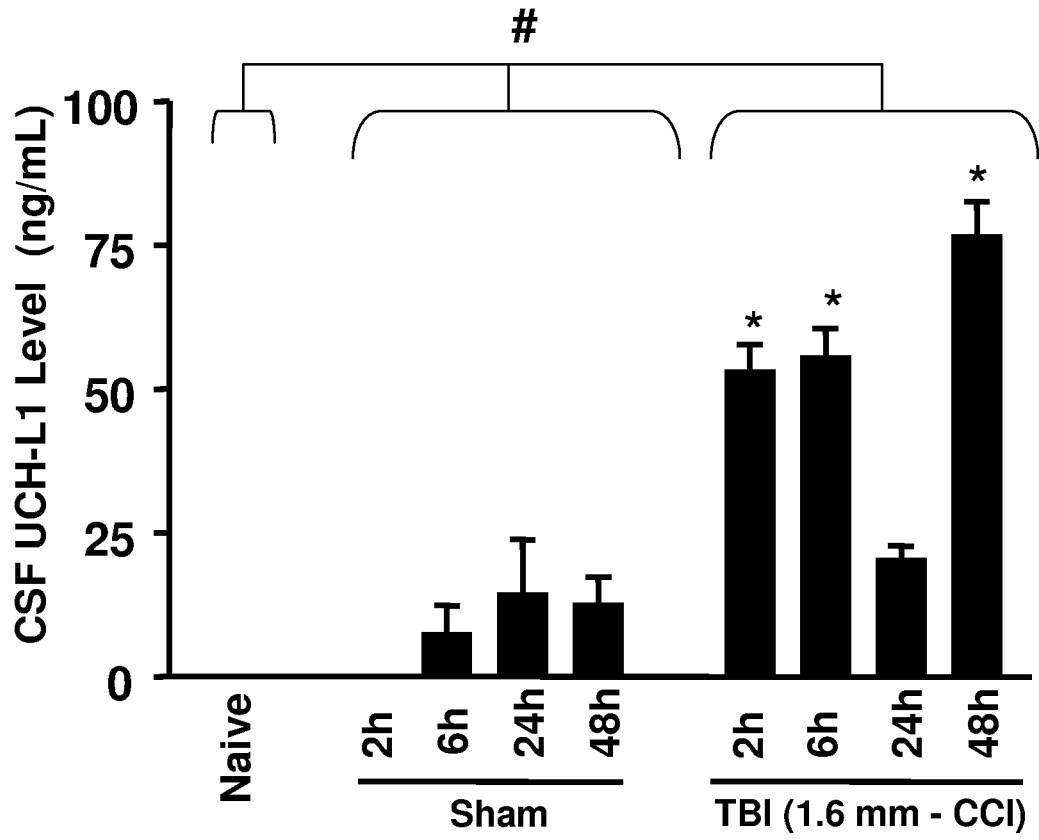


Figure 2

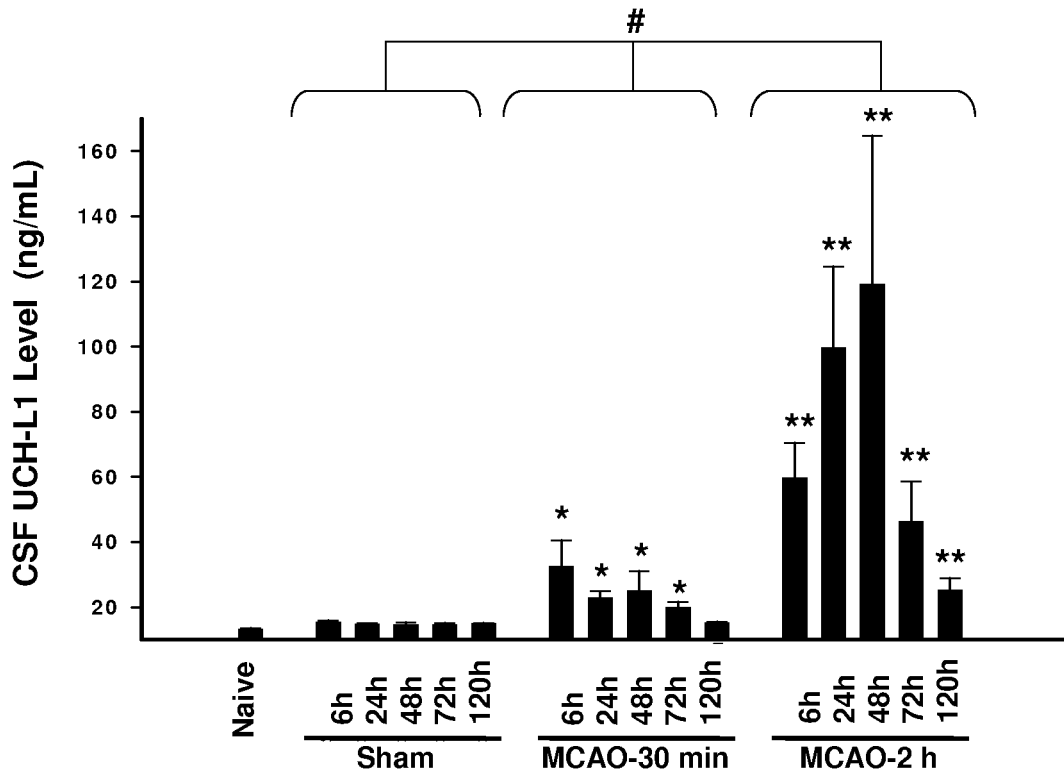


Figure 3

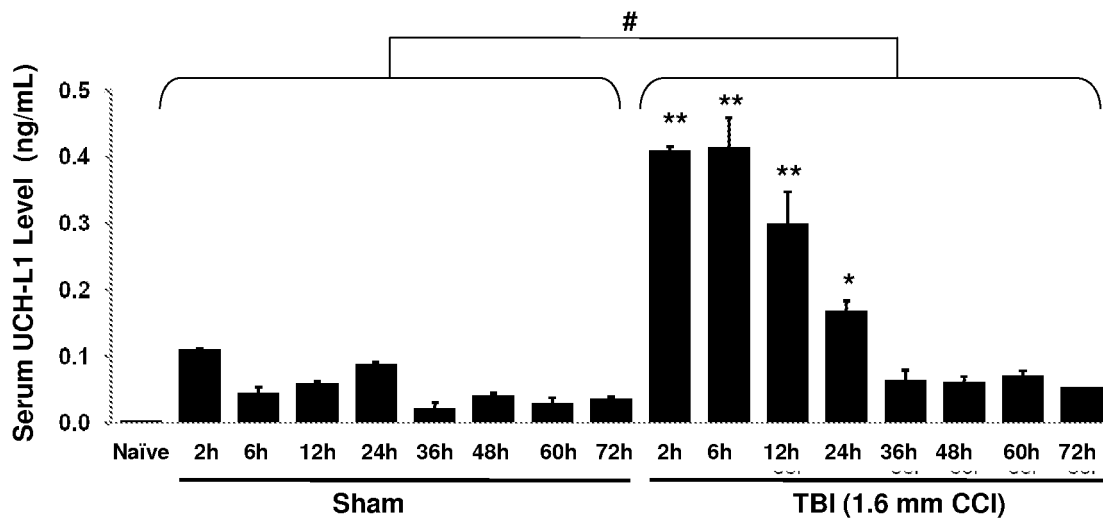


Figure 4

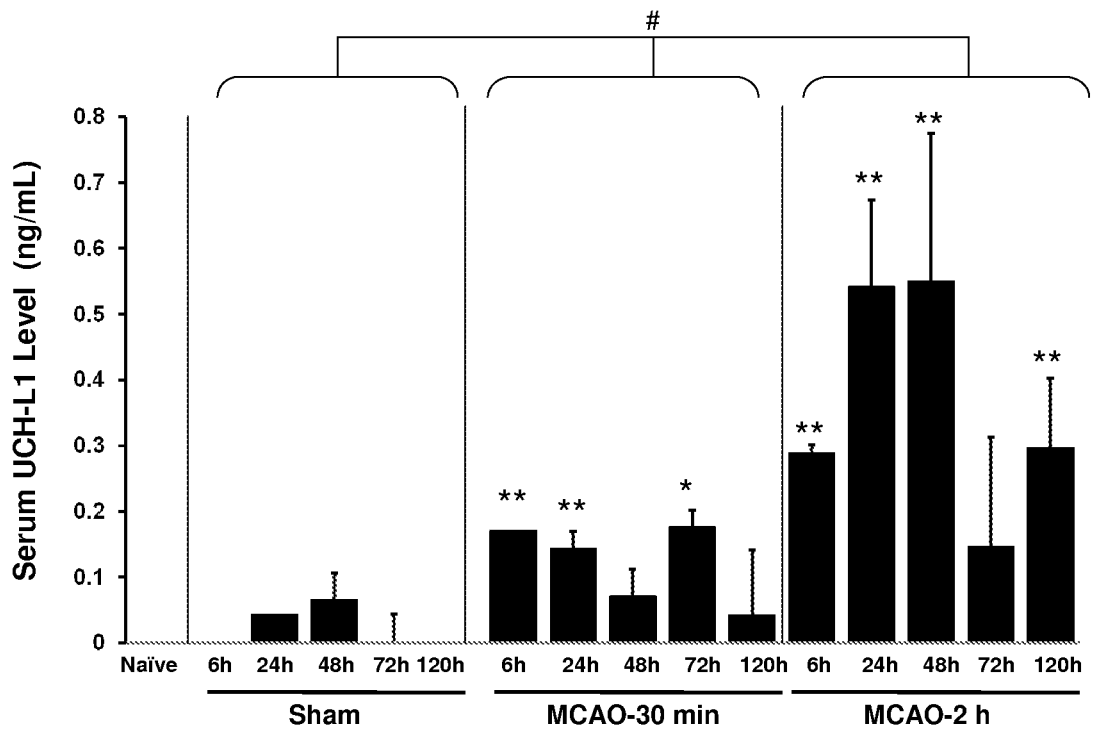
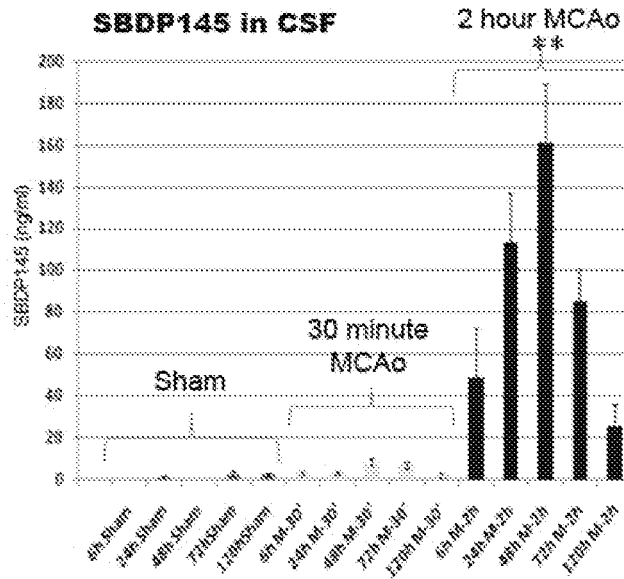


Figure 5

A



B

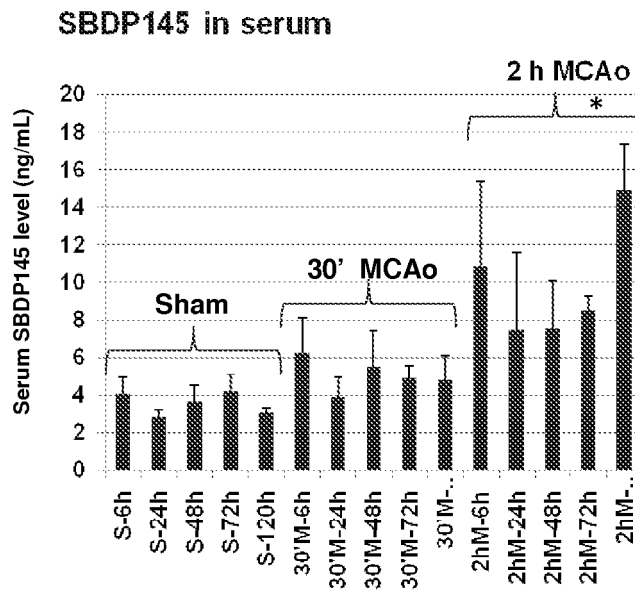


Figure 6

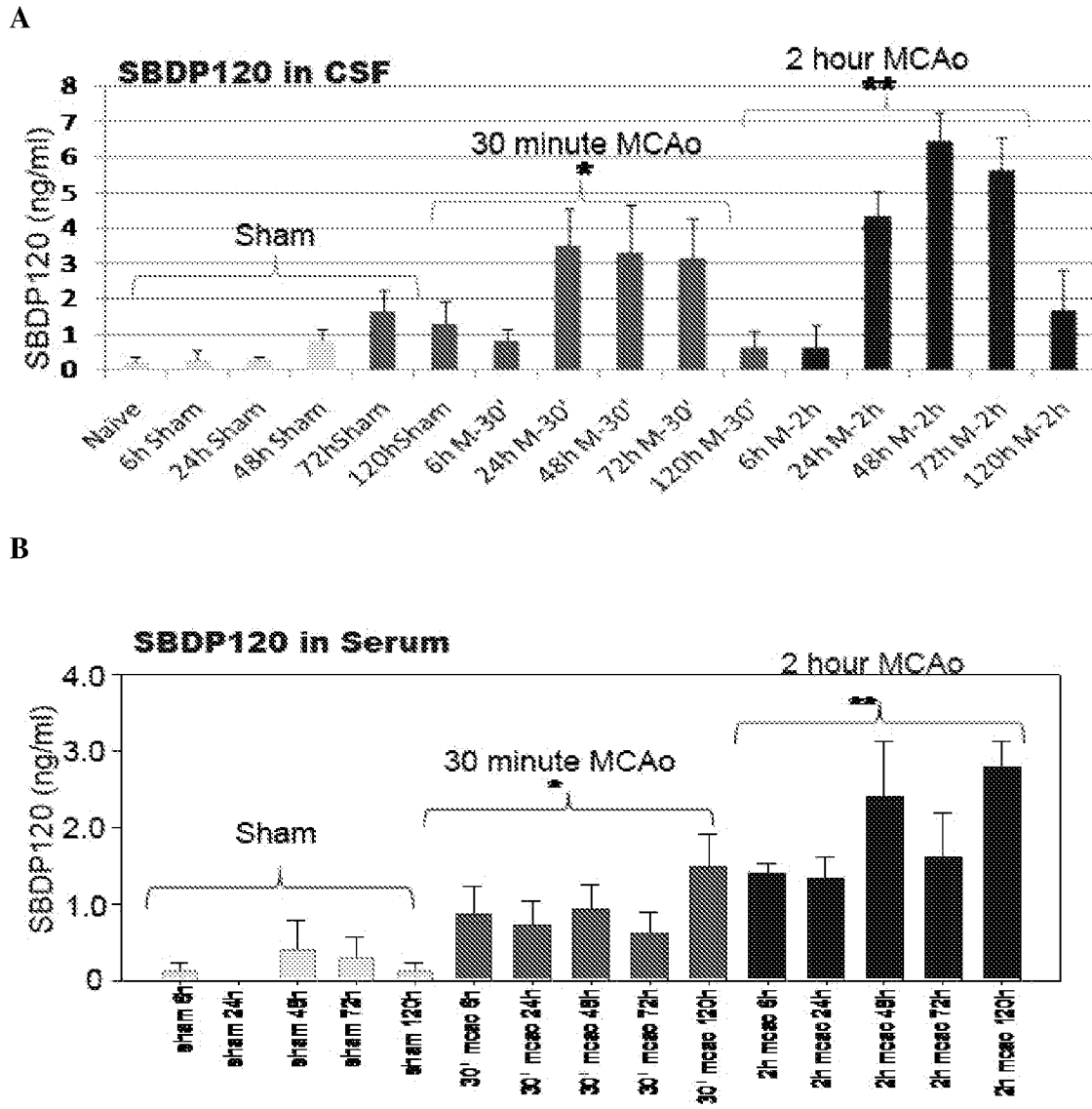


Figure 7

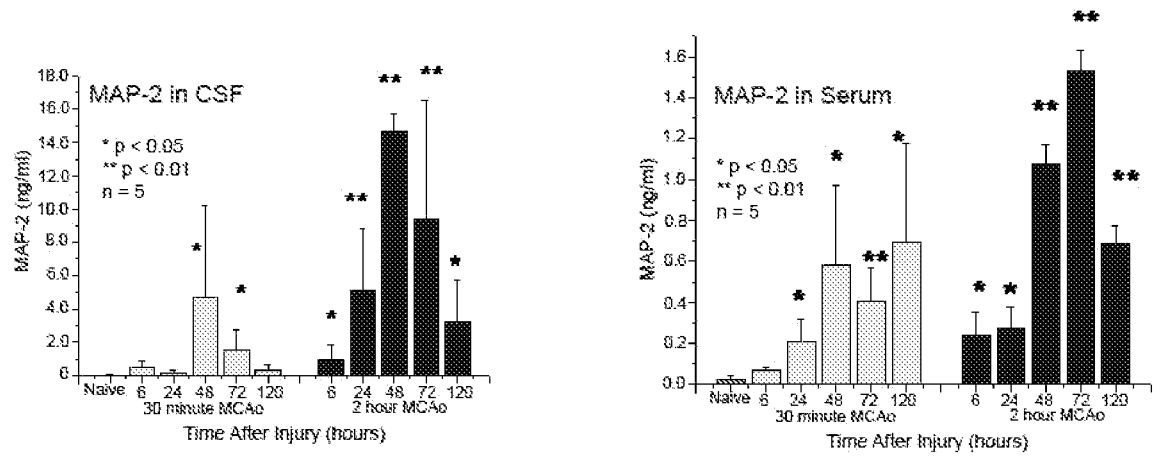
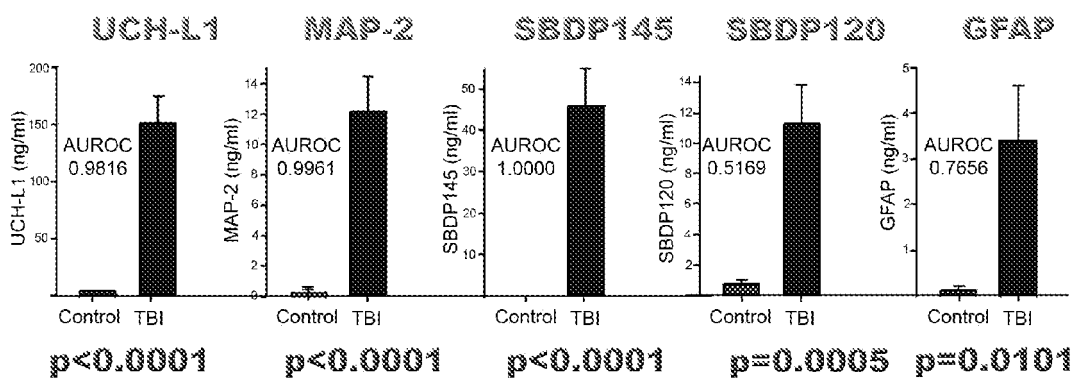


Figure 8

Diagnostic Utility of Biomarkers Using First Available CSF Sample



Time to First Serum Sample

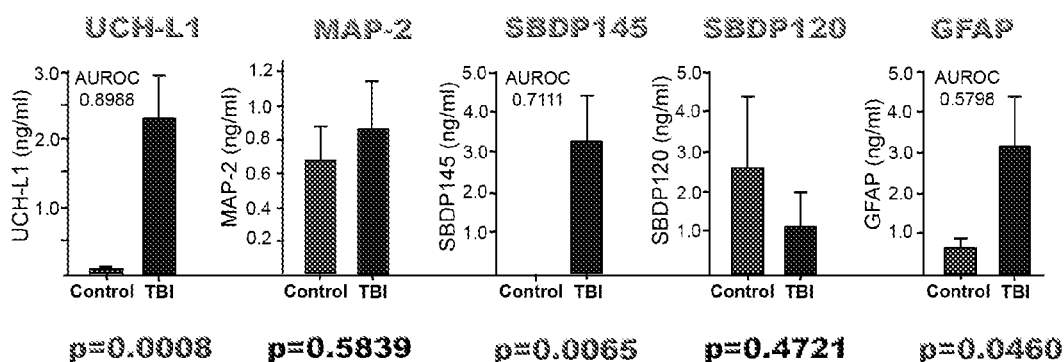
Average	11.2 hours
Std Dev	5.59
Minimum	2
Maximum	24

Sample Sizes

TBI Subjects:	41
Controls	19

Figure 9

Diagnostic Utility of Biomarkers Using First Available Serum Sample



Time to First Serum Sample

Average 10.1 hours
 Std Dev 8.96
 Minimum 2
 Maximum 24

Sample Sizes

TBI Subjects: 45
 Controls (MAP-2, UCH-L1, GFAP) 54
 Controls (SBDP145, SBDP120) 10

Figure 10

Diagnostic Utility of Biomarkers Within First 12 Hours After Injury

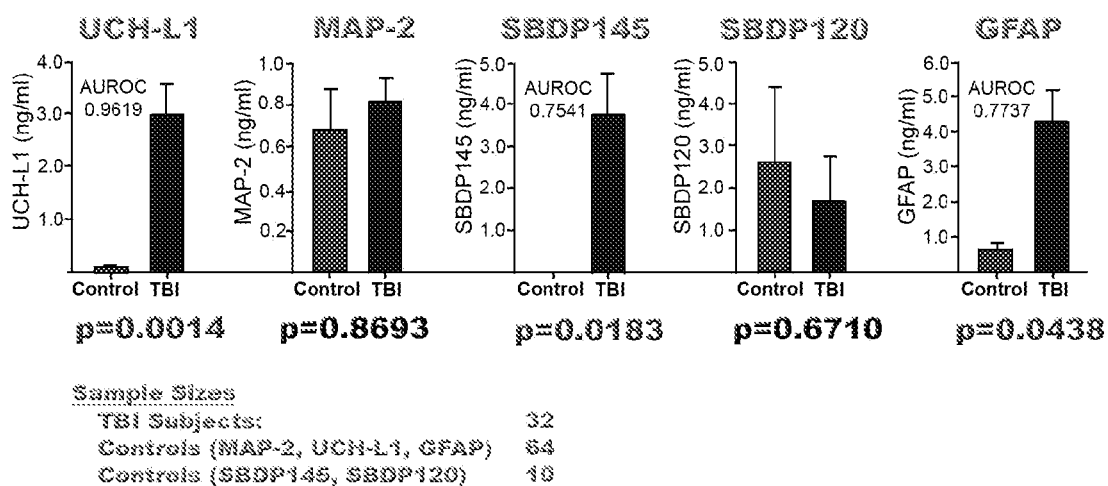


Figure 11

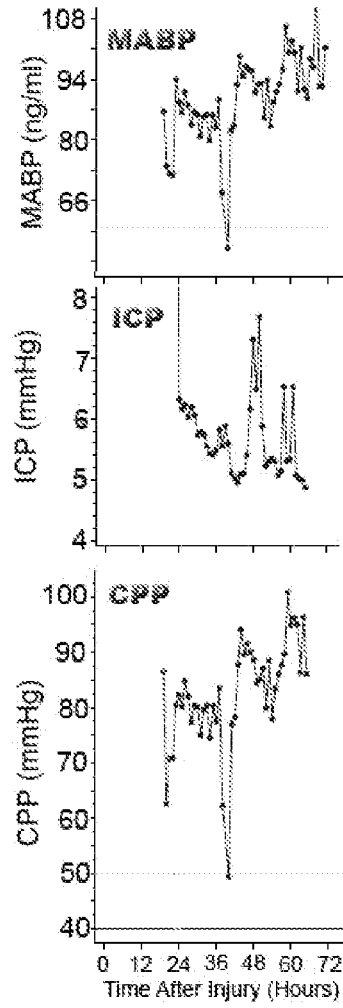


Figure 12

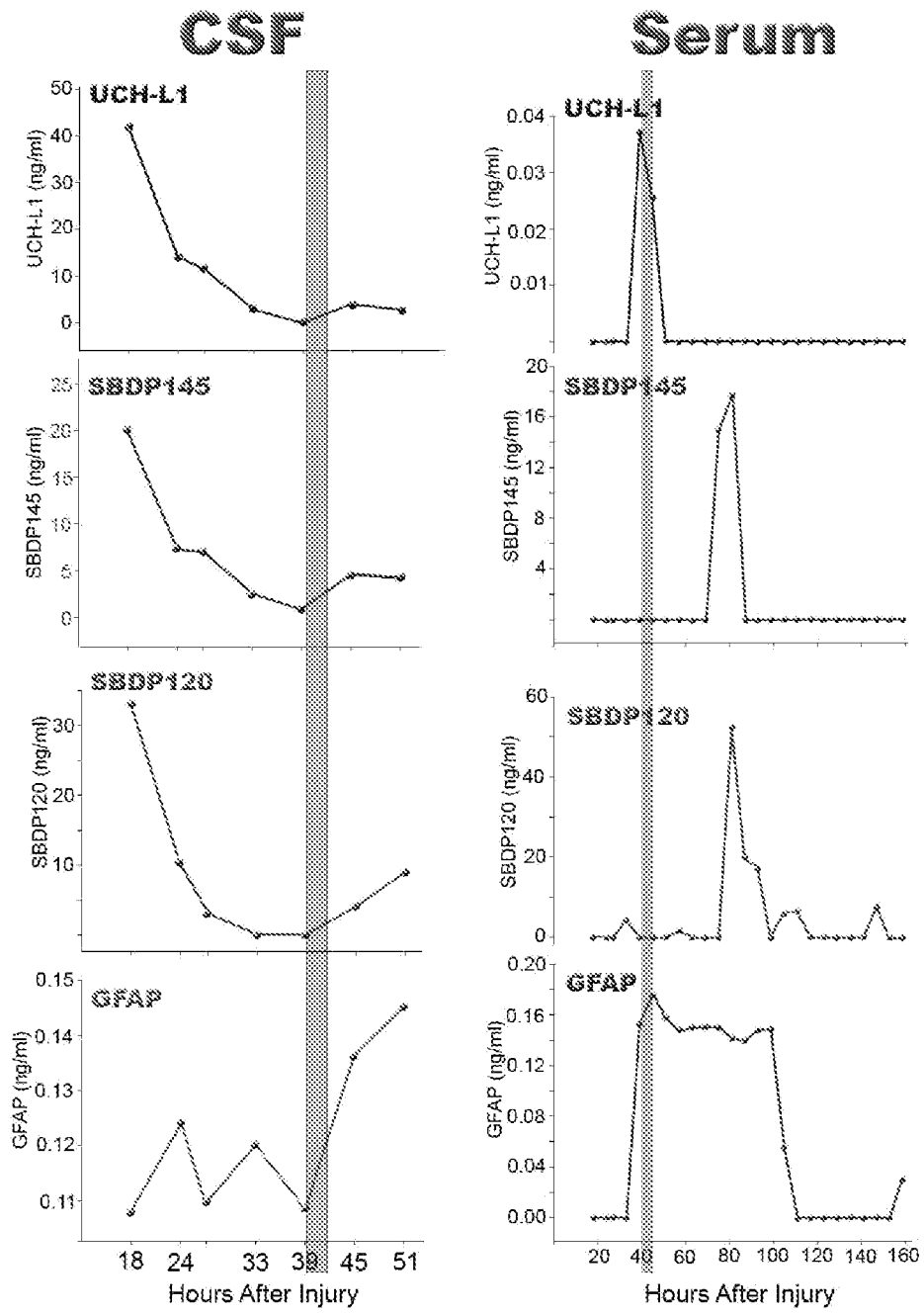


Figure 13

Subject UF-002

CSF

Serum

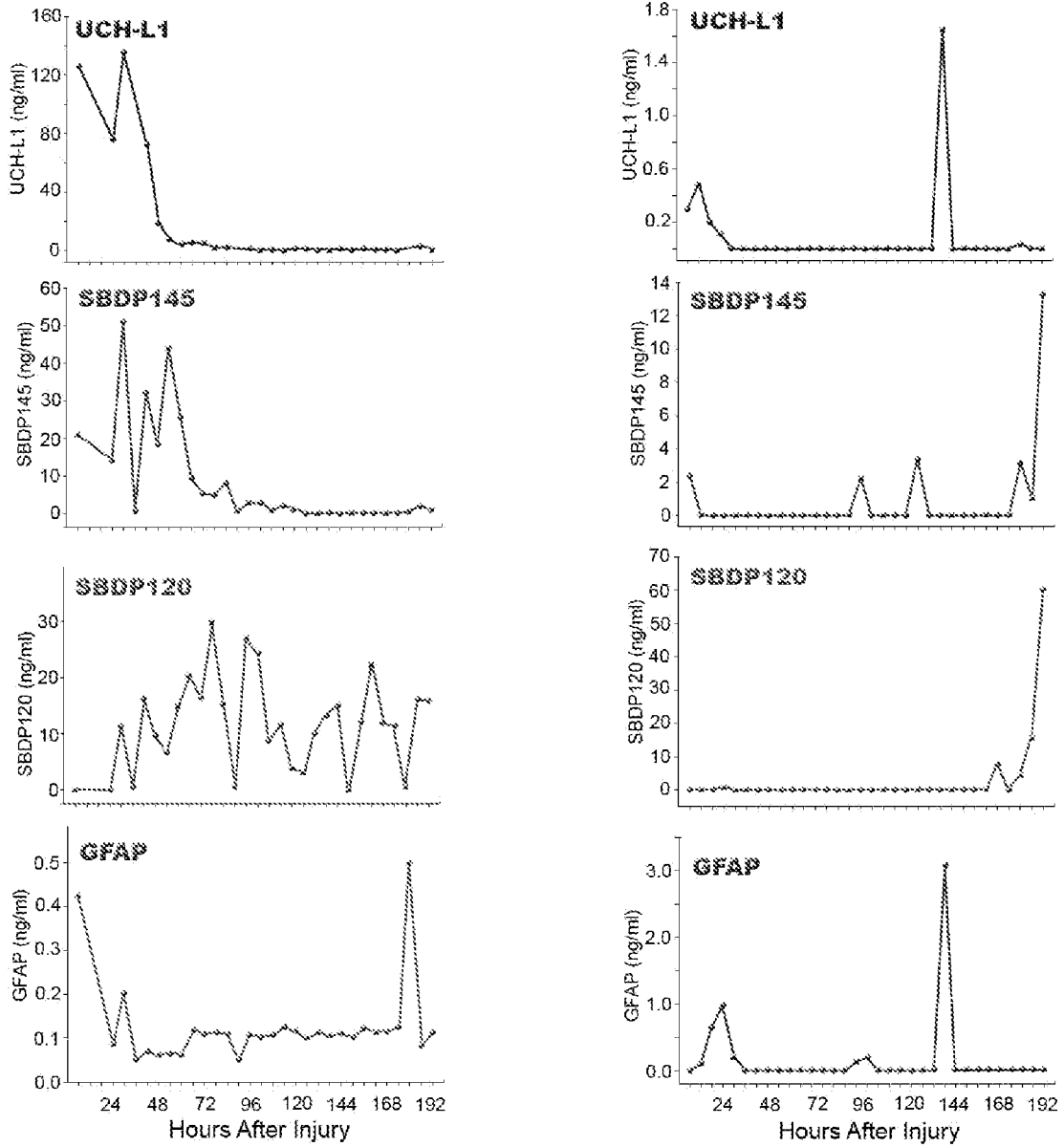


Figure 14

**UCH-L1 – Preclinical Confirmation
CCI Model**

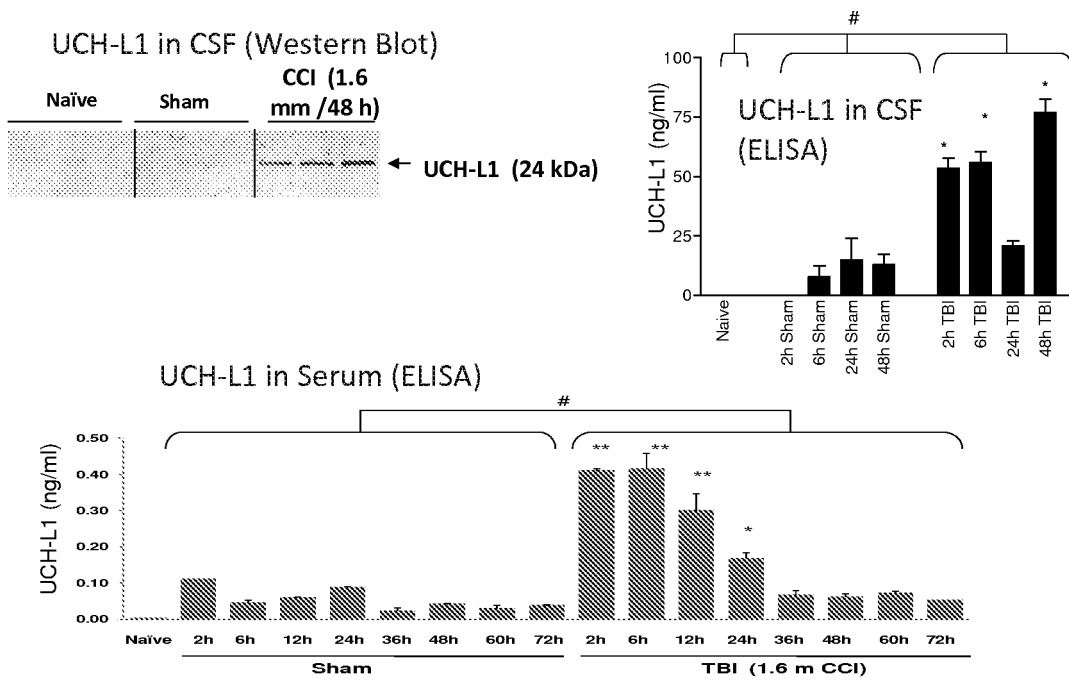


Figure 15

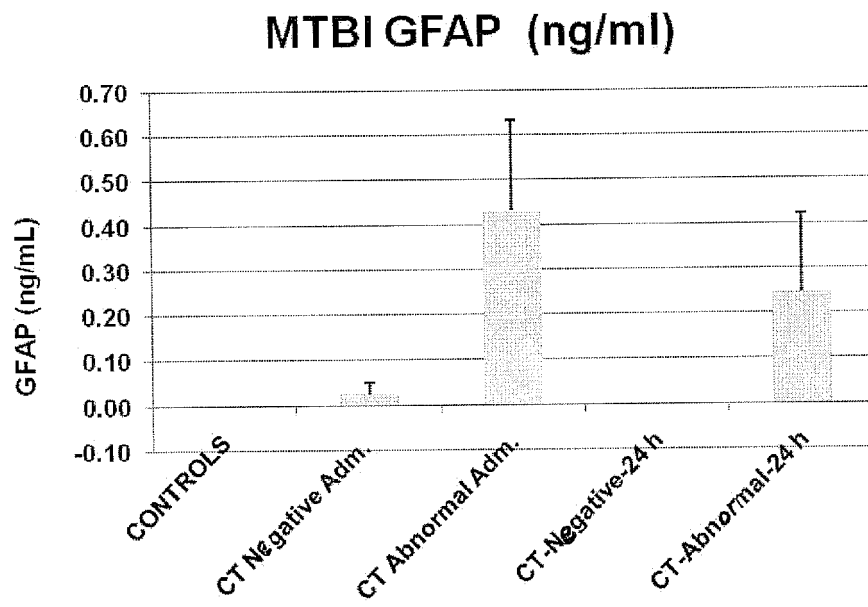


Figure 16

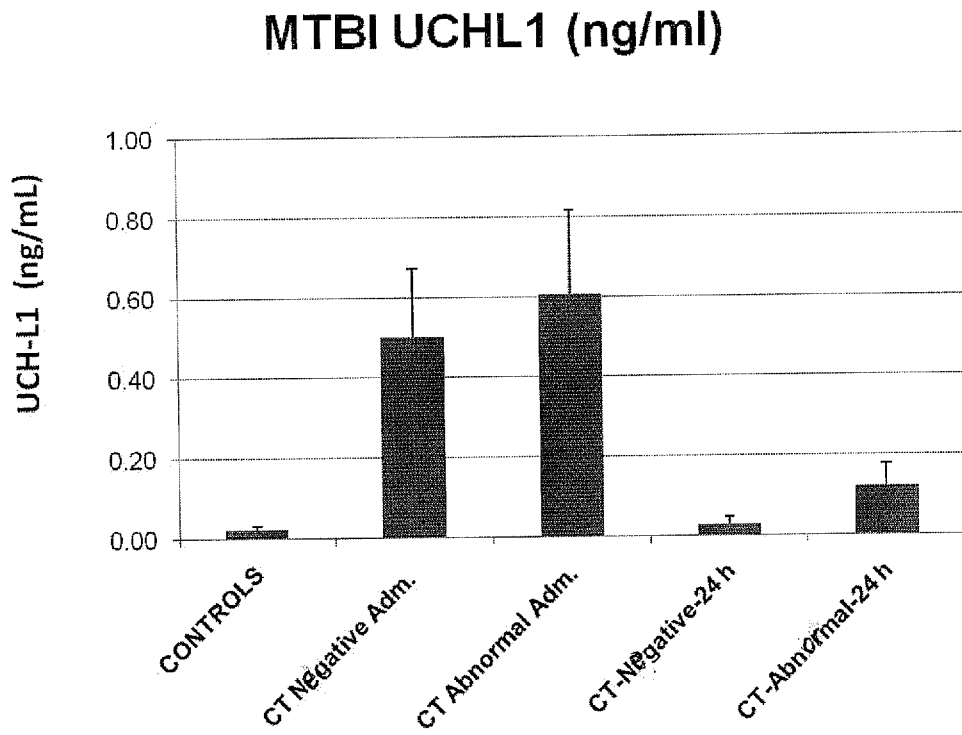
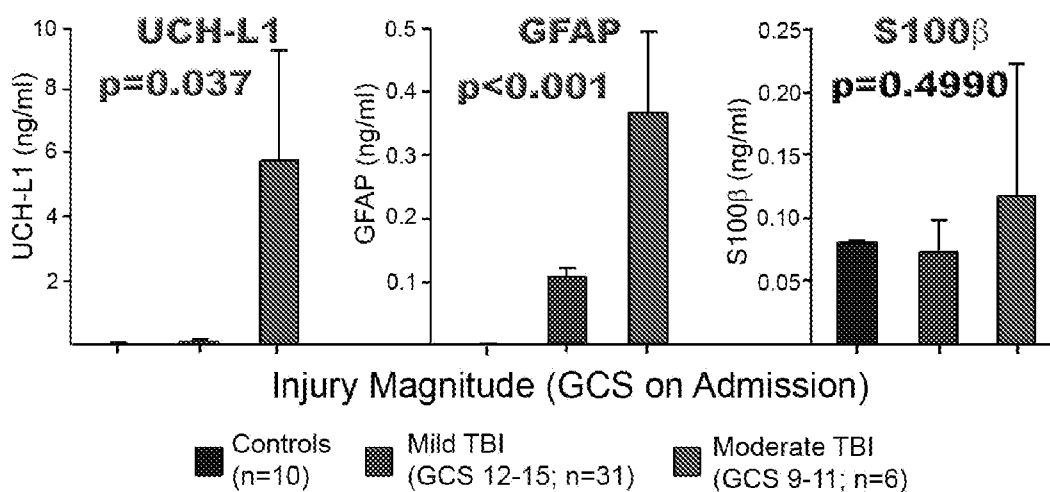


Figure 17

Diagnostic Utility of Biomarkers to Detect Mild and Moderate TBI

1. Relationship to GCS

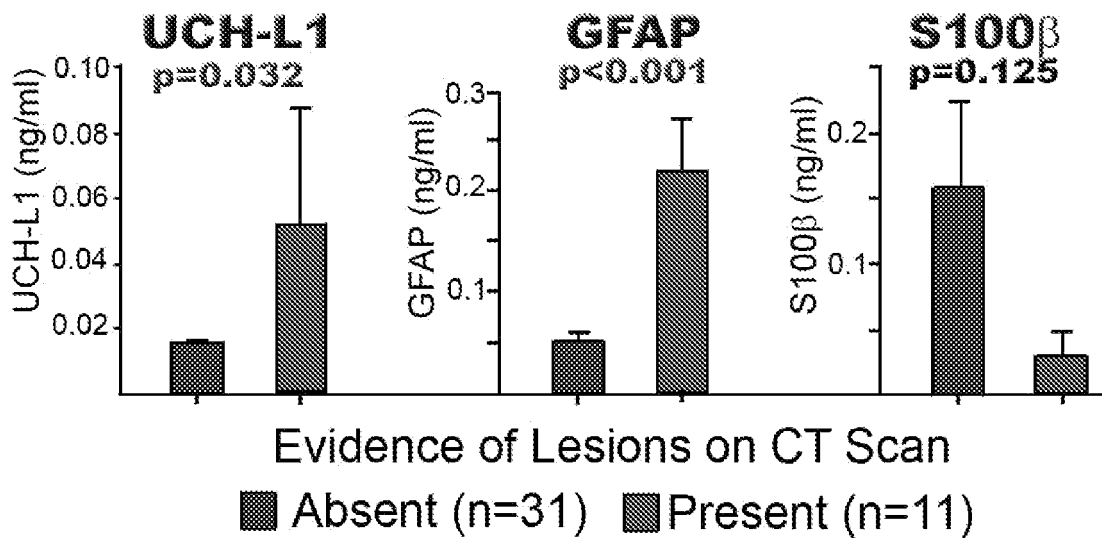


Biomarkers quantified in serum samples taken on Emergency Room admission (within 2 hours of injury)

Figure 18

Diagnostic Utility of Biomarkers to Detect Mild and Moderate TBI

2. Relationship to CT Scan



Biomarkers quantified in serum samples taken on Emergency Room admission (within 2 hours of injury)

Figure 19

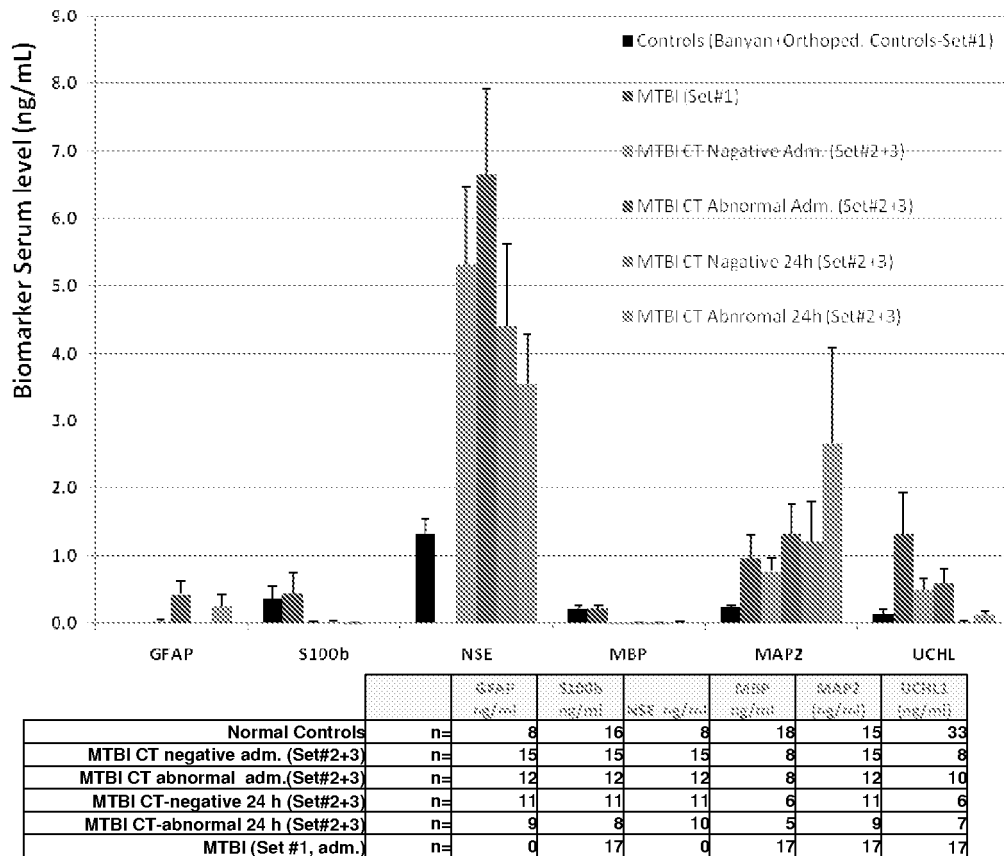


Figure 20

ROC analysis of UCH-L1, GFAP and SBDP145 in human CSF (severe TBI vs. Control)
 A) First 24 hours post-injury

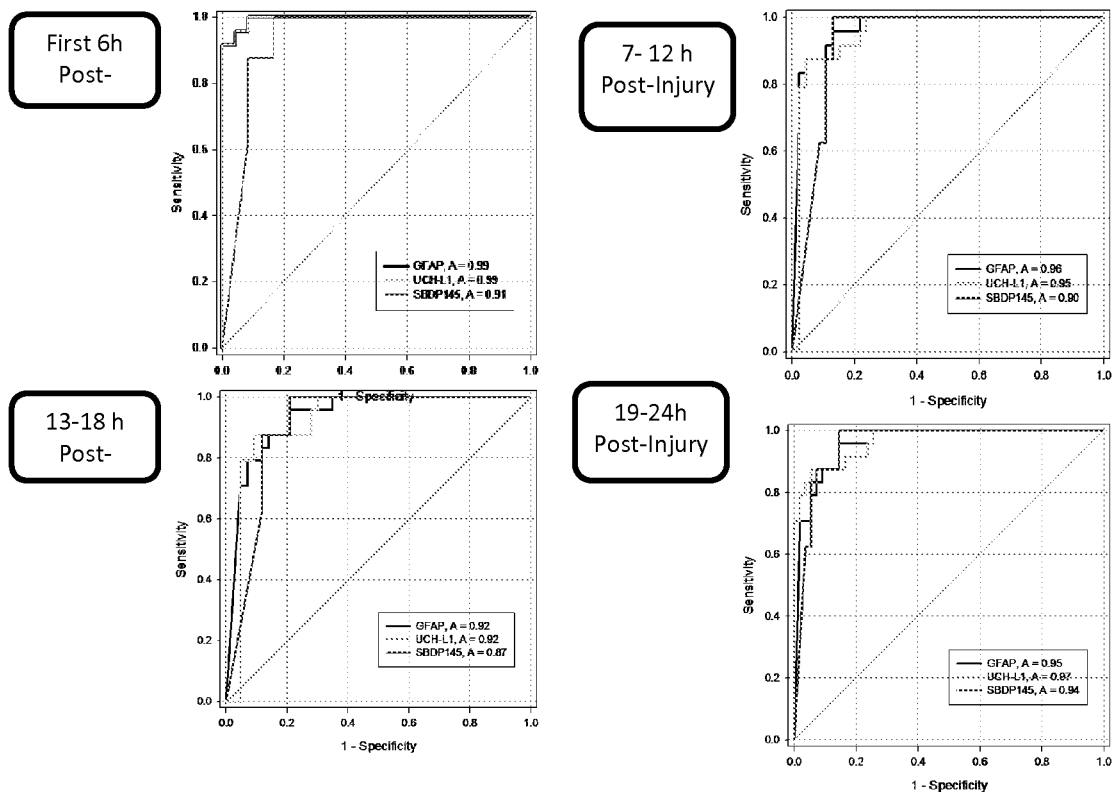


Fig. 21

ROC Curves: Biomarkers in Mild TBI

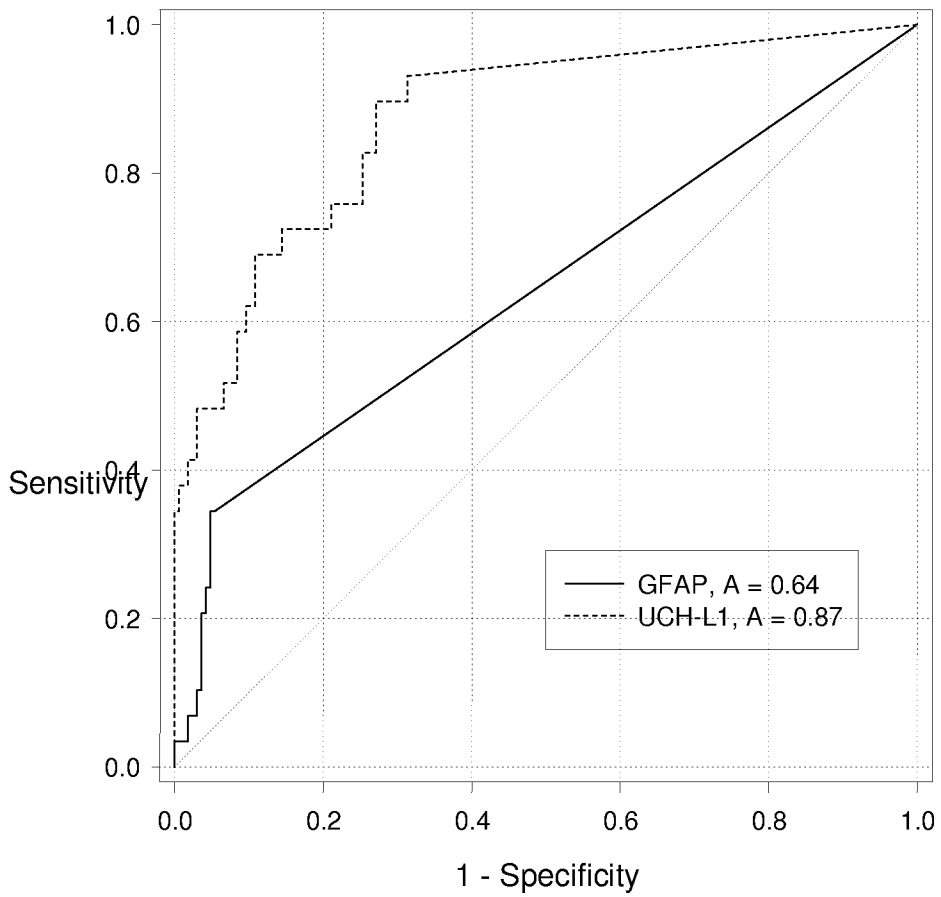


Fig. 22

Elevation of brain injury biomarkers (GFAP, UCH-L1 and MAP2) in plasma in stroke patients.

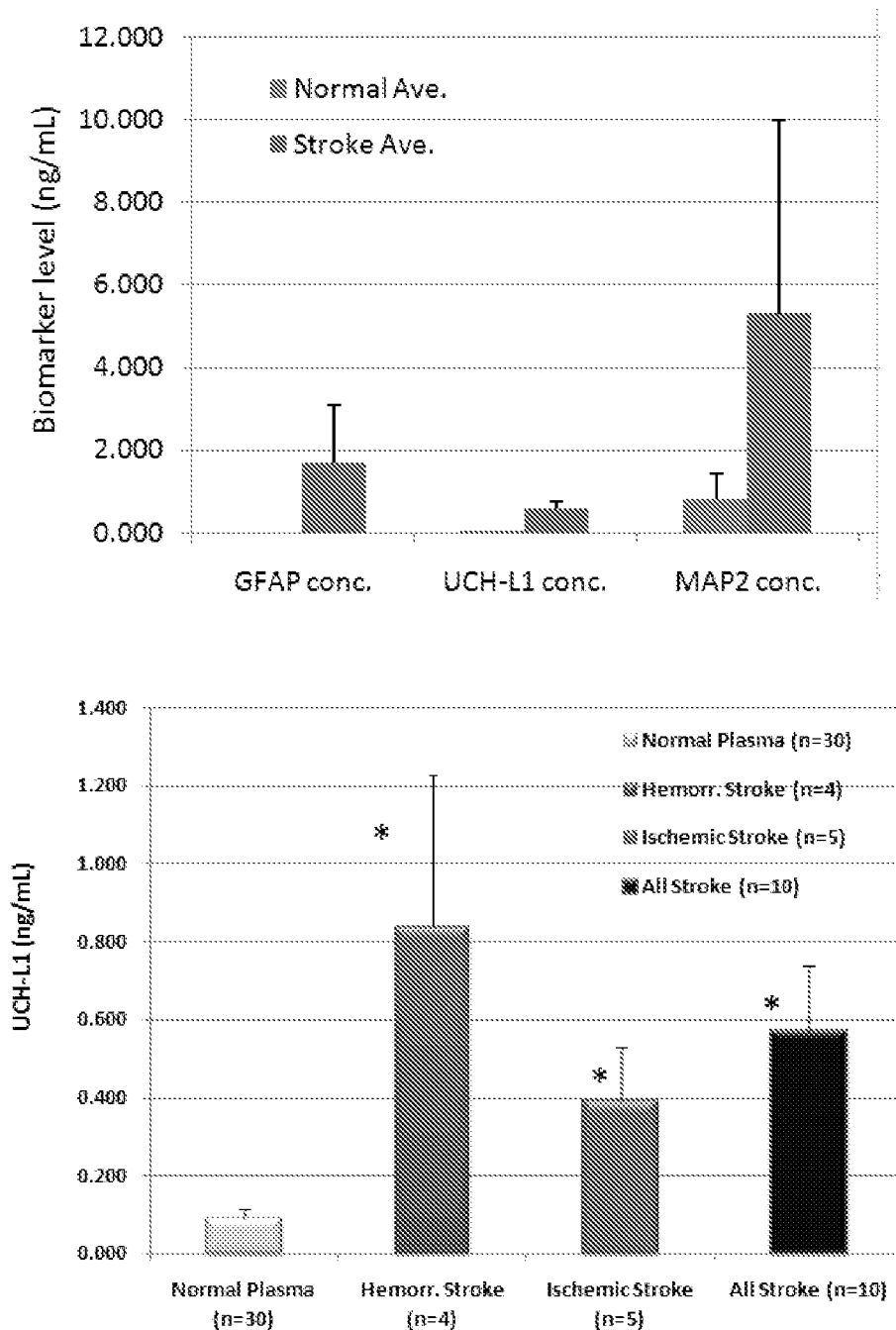


Fig. 23

REFERENCES CITED IN THE DESCRIPTION

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专利名称(译)	生物标志物检测过程和神经病学测定		
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摘要(译)

本发明提供了用于诊断受试者的神经病症的稳健, 定量和可重复的过程和测定。本发明提供了对诸如CSF或血清的生物学流体中的两种或更多种生物标志物的测量, 从而产生协同机制, 用于确定具有异常神经病症的受试者的神经损伤程度, 并用于辨别其子类型或遭受损伤的组织类型。