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(54) **Title:** METHODS FOR AIDING IN THE HYPERACUTE DIAGNOSIS AND DETERMINATION OF TRAUMATIC BRAIN INJURY IN A HUMAN SUBJECT USING EARLY BIOMARKERS

(57) **Abstract:** Disclosed herein are methods that aid in the hyperacute diagnosis and evaluation of a human subject that has sustained or may have sustained an injury to the head, such as mild or moderate, severe, or moderate to severe traumatic brain injury (TBI), using an early biomarker, such as ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) glial fibrillary acidic protein (GFAP), or a combination thereof. Also disclosed here are methods that aid in the hyperacute determination of whether a human subject that has sustained an injury or may have sustained to the head would benefit from and thus receive a head computerized tomography (CT) scan based on the levels of UCH-L1. These methods involve detecting levels of early biomarker, such as ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) glial fibrillary acidic protein (GFAP), or a combination thereof, in samples taken from a human subject at a time point within about 2 hours, such as about 10, 12, or 20 minutes, after the subject has sustained or may have sustained an injury to the head.

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METHODS FOR AIDING IN THE HYPERACUTE DIAGNOSIS AND DETERMINATION OF TRAUMATIC BRAIN INJURY IN A HUMAN SUBJECT USING EARLY BIOMARKERS

RELATED APPLICATION INFORMATION

This application claims priority to U.S. Application No. 62/485,935 filed on April 15, 2017 and U.S. Application No. 62/487,703 filed on April 20, 2017, the contents of each of which are herein incorporated by reference.

TECHNICAL FIELD

[0001] The present invention relates to methods of aiding in the hyperacute diagnosis and evaluation of a human subject that has sustained or may have sustained an injury to the head, such as mild or moderate to severe traumatic brain injury (TBI), or a mild, moderate, severe, or moderate to severe TBI, by detecting levels of an early biomarker, such as ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) glial fibrillary acidic protein (GFAP), or a combination thereof, in samples taken from a human subject at time points within about 2 hours, such as about 10, 12, or 20 minutes, after the subject has sustained or may have sustained an injury to the head.

BACKGROUND

[0002] More than 5 million mild traumatic brain injuries (TBIs) occur each year in the United States alone. Currently, there is no simple, objective, accurate measurement available to help in patient assessment. In fact, much of TBI evaluation and diagnosis is based on subjective data. Unfortunately, objective measurements such as head CT and Glasgow Coma Score (GCS) are not very comprehensive or sensitive in evaluating mild TBI. Moreover, head CT is unrevealing for the vast majority of the time for mild TBI, is expensive, and exposes the patient to unnecessary radiation. Additionally, a negative head CT does not mean the patient has been cleared from having a concussion; rather it just means certain interventions, such as surgery is not warranted. Clinicians and patients need objective, reliable information to accurately evaluate this condition to promote appropriate triage and recovery. To date, limited data have been available for the use of UCH-L1 and GFAP in the hyperacute care setting (very early acute time points after injury) to aid in patient evaluation and management.

[0003] Mild TBI or concussion is much harder to objectively detect and presents an everyday challenge in emergency care units globally. Concussion usually causes no gross pathology, such as hemorrhage, and no abnormalities on conventional computed tomography scans of the brain, but rather rapid-onset neuronal dysfunction that resolves in a spontaneous manner over a few days to a few weeks. Approximately 15% of mild TBI patients suffer persisting cognitive dysfunction. There is an unmet need for mild TBI victims on scene, in emergency rooms and clinics, in the sports area and in military activity (*e.g.*, combat).

[0004] Current algorithms for assessment of the severity of brain injury include Glasgow Coma Scale score and other measures. These measures may at times be adequate for relating acute severity but are insufficiently sensitive for subtle pathology which can result in persistent deficit. GCS and other measures also do not enable differentiation among types of injury and may not be adequate. Thus patients grouped into a single GCS level entering a clinical trial may have vastly heterogeneous severity and type of injury. Because outcomes also vary accordingly, inappropriate classification undermines the integrity of a clinical trial. Improved classification of injury will enable more precise delineation of disease severity and type for TBI patients in clinical trials.

[0005] Additionally, current brain injury trials rely on outcome measures such as Glasgow Outcome Scale Extended, which capture global phenomena but fail to assess for subtle differences in outcome. Thus 30 consecutive trials for brain injury therapeutics have failed. Sensitive outcome measures are needed to determine how well patients have recovered from brain injury in order to test therapeutics and prophylactics.

SUMMARY

[0006] In one embodiment, the present disclosure is directed to a method of aiding in the diagnosis and evaluation of a human subject that has sustained or may have sustained an injury to the head. The method comprises: performing an assay on a sample obtained from the subject within about 2 hours after a suspected injury to the head to measure or detect a level of an early biomarker in the sample, said early biomarker comprising ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), glial fibrillary acidic protein (GFAP), or a combination thereof; and determining whether the subject has sustained a mild, moderate, severe, or a moderate to severe traumatic brain injury (TBI), wherein the subject is determined as having (1) a moderate, severe, or a moderate to severe traumatic brain injury when the level of the early biomarker in the sample is higher than a reference level of the early biomarker or (2) a mild traumatic brain injury when the level of the early biomarker in the sample is lower than

a reference level of the early biomarker. In some embodiments, the early biomarker comprises UCH-L1. In other embodiments, the early biomarker comprises GFAP. In yet other embodiments, the biomarker comprises the detection of both UCH-L1 and GFAP. Depending on the clinical circumstances of a subject, the skilled person will select the appropriate early biomarker to be employed in the methods described herein. For example, in some instances based on the clinical circumstances of the subject, the skilled person will assay only for the biomarker UCH-L1. In other instances, based on the clinical circumstances, the skilled person will assay only for the biomarker GFAP. In other instances, based on the clinical circumstances, the skilled person will assay for both the biomarkers UCH-L1 and GFAP. UCH-L1, GFAP, or the combination of UCH-L1 and GFAP may be assayed alone, or, may be combined with an assessment of one or more other biomarkers. In other aspects, the methods described herein may be performed more than once on a subject with the skilled person assaying for different early biomarkers and combinations thereof depending on the clinical circumstances of the subject.

[0007] In another embodiment, the present disclosure is directed to a method of evaluating a human subject that has sustained or may have sustained an injury to the head. The method comprises: performing an assay on a sample obtained from the subject within about 2 hours after a suspected injury to the head to measure a level of an early biomarker in the sample, said early biomarker comprising ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), glial fibrillary acidic protein (GFAP), or a combination thereof; and determining whether the subject has sustained a mild, moderate, severe, or a moderate to severe traumatic brain injury (TBI), wherein the subject is determined as having (1) a moderate, severe, or a moderate to severe traumatic brain injury when the level of the early biomarker in the sample is higher than a reference level of the early biomarker or (2) a mild traumatic brain injury when the level of the early biomarker in the sample is lower than a reference level of the early biomarker. In some embodiments, the early biomarker comprises UCH-L1. In other embodiments, the early biomarker comprises GFAP. In yet other embodiments, the biomarker comprises the detection of both UCH-L1 and GFAP. Depending on the clinical circumstances of a subject, the skilled person will select the appropriate early biomarker to be employed in the methods described herein. For example, in some instances based on the clinical circumstances of the subject, the skilled person will assay only for the biomarker UCH-L1. In other instances, based on the clinical circumstances, the skilled person will assay only for the biomarker GFAP. In other instances, based on the clinical circumstances, the skilled person will assay for the biomarkers UCH-L1 and GFAP. In other aspects, the

methods described herein may be performed more than once on a subject with the skilled person assaying for different early biomarkers and combinations thereof depending on the clinical circumstances of the subject.

[0008] In some aspects of the above-described methods, the subject may have received a Glasgow Coma Scale score before or after the assay is performed. In some aspects, the subject may be suspected of having a traumatic brain injury based on a Glasgow Coma Scale score that was previously performed (meaning prior to the assay being performed). For example, depending upon a subject's medical condition, a Glasgow Coma Scale score may be assessed shortly after the subject arrives at an emergency room, trauma center, or other site in order to assess and/or evaluate whether the subject has a TBI. Such a Glasgow Coma Scale score may be provided prior to the assay being performed to confirm and determine whether or not the subject has a mild or moderate to severe TBI. After the assay is performed, one or more subsequent Glasgow Coma Scale scores can be performed based on the results of the assay as part of the physician's (or other medical personnel's) management of the TBI (such as, for example, to determine whether surgical and/or pharmacological intervention may be required). In other aspects, the subject may not have received a Glasgow Coma Scale score before the assay is performed.

[0009] In fact, in some aspects, the subject may be suspected as having mild TBI based on the Glasgow Coma Scale score. In other aspects, the subject may be suspected of having a moderate TBI based on the Glasgow Coma Scale score. In other aspects, the subject may be suspected of having a severe TBI based on the Glasgow Coma Scale Score. In other aspects, the subject may be suspect as having a moderate to severe TBI based on the Glasgow Coma scale score. In other aspects, the reference level of GFAP or the reference level of UCH-L1 correlate with or correspond to a Glasgow Coma Scale score of 13-15 (a mild TBI). In other aspects, the reference level of GFAP or the reference level of UCH-L1 correlate or correspond to a Glasgow Coma Scale score of 3-8 (a severe TBI). In other aspects, the reference level of GFAP or the reference level of UCH-L1 correlate or correspond to a Glasgow Coma Scale score of 9-13 (a moderate TBI). In other aspects, the reference level of GFAP or the reference level of UCH-L1 correlate with or correspond to a Glasgow Coma Scale score of 3-12 (a moderate to severe TBI).

[0010] In some aspects in the above-described methods, the reference level is correlated or corresponds to level in control subjects that have not sustained a head injury.

In the above-described methods, the reference level can comprise a recited value or range of values. For example, in one aspect, the reference level for GFAP can be about 9.0 pg/mL,

about 11.7 pg/mL or about 42.0 pg/mL of a biological sample, such as, for example, a whole blood sample, a serum sample, or a plasma sample. In another aspect, the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL of a biological sample, such as, for example, a whole blood sample, a serum sample, or a plasma sample. In yet another aspect, the reference level for UCH-L1 is about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL of a biological sample, such as, for example, a whole blood sample, a serum sample or a plasma sample. In yet still a further aspect, the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL of a biological sample, such as, for example, a whole blood sample, a serum sample or a plasma sample. In another aspect, the reference level for GFAP can be about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL of a whole blood sample. In another aspect, the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL of a whole blood sample. In yet another aspect, the reference level for UCH-L1 is about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL of a whole blood sample. In yet still a further aspect, the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL of a whole blood sample. In another aspect, the reference level for GFAP can be about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL of a serum sample. In another aspect, the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL of a serum sample. In yet another aspect, the reference level for UCH-L1 is about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL of a serum sample. In yet still a further aspect, the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL of a serum sample. In another aspect, the reference level for GFAP can be about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL of a plasma sample. In another aspect, the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL of a plasma sample. In yet another aspect, the reference level for UCH-L1 is about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL of a plasma sample. In yet still a further aspect, the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL of a plasma sample.

[0011] In still yet a further aspect, the reference level is (a) determined by an assay having a sensitivity of between at least about 65% to 100% and a specificity of between at least about 30% to 100%; (b) determined by an assay having a sensitivity of at least about 99% and a specificity of at least about 75%; (c) between at least about 5 pg/mL to about 3500 pg/mL; or (d) between at least about 10 pg/mL to about 1000 pg/mL of a biological sample, such as, for example, a whole blood sample, a serum sample or a plasma sample. In still yet a further aspect, the reference level for UCH-L1 is from at least about 70 pg/mL to about 3500 pg/mL and the reference level for GFAP is from at least about 5 pg/mL to about 2000 pg/mL of a biological sample, such as, for example a whole blood sample, a serum sample or a plasma sample. In still yet a further aspect, the reference level is (a) determined by an assay having a sensitivity of between at least about 65% to 100% and a specificity of between at least about 30% to 100%; (b) determined by an assay having a sensitivity of at least about 99% and a specificity of at least about 75%; (c) between at least about 5 pg/mL to about 3500 pg/mL; or (d) between at least about 10 pg/mL to about 1000 pg/mL of a whole blood sample. In still yet a further aspect, the reference level for UCH-L1 is from at least about 70 pg/mL to about 3500 pg/mL and the reference level for GFAP is from at least about 5 pg/mL to about 2000 pg/mL of a whole blood sample. In still yet a further aspect, the reference level is (a) determined by an assay having a sensitivity of between at least about 65% to 100% and a specificity of between at least about 30% to 100%; (b) determined by an assay having a sensitivity of at least about 99% and a specificity of at least about 75%; (c) between at least about 5 pg/mL to about 3500 pg/mL; or (d) between at least about 10 pg/mL to about 1000 pg/mL of a serum sample. In still yet a further aspect, the reference level for UCH-L1 is from at least about 70 pg/mL to about 3500 pg/mL and the reference level for GFAP is from at least about 5 pg/mL to about 2000 pg/mL of a serum sample. In still yet a further aspect, the reference level is (a) determined by an assay having a sensitivity of between at least about 65% to 100% and a specificity of between at least about 30% to 100%; (b) determined by an assay having a sensitivity of at least about 99% and a specificity of at least about 75%; (c) between at least about 5 pg/mL to about 3500 pg/mL; or (d) between at least about 10 pg/mL to about 1000 pg/mL of a plasma sample. In still yet a further aspect, the reference level for UCH-L1 is from at least about 70 pg/mL to about 3500 pg/mL and the reference level for GFAP is from at least about 5 pg/mL to about 2000 pg/mL of a plasma sample.

[0012] In yet another aspect in the above-described methods, UCH-L1 can be measured by:

A. contacting the sample, either simultaneously or sequentially, in any order with:

(1) a UCH-L1-capture antibody, which binds to an epitope on UCH-L1 or UCH-L1 fragment to form a UCH-L1-capture antibody-UCH-L1 antigen complex, and

(2) a UCH-L1-detection antibody which includes a detectable label and binds to an epitope on UCH-L1 that is not bound by the UCH-L1-capture antibody, to form a UCH-L1 antigen-UCH-L1-detection antibody complex,

such that a UCH-L1-capture antibody-UCH-L1 antigen-UCH-L1-detection antibody complex is formed, and

B. measuring the amount or concentration of UCH-L1 in the sample based on the signal generated by the detectable label in the UCH-L1-capture antibody-UCH-L1 antigen-UCH-L1-detection antibody complex.

[0013] In yet another aspect in the above-described methods, GFAP can be measured by:

A. contacting the sample, either simultaneously or sequentially, in any order with:

(1) a GFAP-capture antibody, which binds to an epitope on GFAP or GFAP fragment to form a GFAP-capture antibody-GFAP antigen complex, and

(2) a GFAP-detection antibody which includes a detectable label and binds to an epitope on GFAP that is not bound by the GFAP-capture antibody, to form a GFAP antigen-GFAP-detection antibody complex,

such that a GFAP-capture antibody-GFAP antigen-GFAP-detection antibody complex is formed, and

B. measuring the amount or concentration of GFAP in the sample based on the signal generated by the detectable label in the GFAP-capture antibody-GFAP antigen-GFAP-detection antibody complex.

[0014] In the above-described methods, the sample can be taken within about 5 minutes after a suspected injury to the head. Alternatively, the sample can be taken within about 10 minutes of a suspected injury to the head. Alternatively, the sample can be taken within about 12 minutes of a suspected injury to the head. Alternatively, the sample can be taken within 15 minutes of a suspected injury to the head. Alternatively, the sample can be taken within about 20 minutes of a suspected injury to the head. Alternatively, the sample can be taken within 30 minutes of a suspected injury to the head. Alternatively, the sample can be taken within 60 minutes of a suspected injury to the head. Alternatively, the sample can be taken within 90 minutes of a suspected injury to the head.

[0015] In one aspect, using the above-described methods, the subject is assessed or evaluated as having a mild TBI. In another aspect, using the above-described methods, the subject is assessed or evaluated as having a moderate TBI. In yet still another aspect, using the above-described methods, the subject is assessed or evaluated as having a severe TBI. In another aspect, using the above-described methods, the subject is assessed or evaluated as having a moderate to severe TBI. In yet still a further aspect, using the above-described methods, the subject is assessed or evaluated as not having a TBI.

[0016] The above-described methods can further comprise treating a human subject assessed or evaluated as having mild, moderate, severe, or moderate to severe TBI with a treatment for TBI (e.g., a surgical treatment, a therapeutic treatment, or combinations thereof). Any such treatment known in the art and described further herein can be used. Moreover, in a further aspect, any subject being treated for TBI can also, optionally, be monitored during and after any course of treatment. Alternatively, said methods can further comprise monitoring a subject assessed as having mild, moderate, severe, or moderate TBI (such as those, who as of yet, may not be receiving any treatment).

[0017] In the above-described methods, the sample can be selected from the group consisting of a whole blood sample, a serum sample, a cerebrospinal fluid sample, and a plasma sample. In some embodiments, the sample is a whole blood sample. In some embodiments, the sample is a plasma sample. In yet other embodiments, the sample is a serum sample. Such a sample can be obtained in a variety of ways. For example, the sample can be obtained after the subject sustained a head injury caused by physical shaking, blunt impact by an external mechanical or other force that results in a closed or open head trauma, one or more falls, explosions or blasts or other types of blunt force trauma. Alternatively, the sample can be obtained after the subject has ingested or been exposed to a chemical, toxin or combination of a chemical and toxin. Examples of chemicals or toxins are fire, mold, asbestos, a pesticide, an insecticide, an organic solvent, a paint, a glue, a gas, an organic metal, a drug of abuse or one or more combinations thereof. Still further, the sample can be obtained from a subject that suffers from an autoimmune disease, a metabolic disorder, a brain tumor, hypoxia, a virus, meningitis, hydrocephalus or combinations thereof.

[0018] Any of the above-described methods can be carried out on any human subject without regard to factors selected from the group consisting of the human subject's clinical condition, the human subject's laboratory values, the human subject's classification as suffering from mild or moderate to severe TBI, the human subject's exhibition of low or high

levels of UCH-L1, GFAP and or UCH-L1 and GFAP, and the timing of any event wherein the human subject may have sustained head injury.

[0019] In the above-described methods, the assay is an immunoassay. In some embodiments, the assay is a point-of-care assay. In yet other embodiments, the assay is a clinical chemistry assay. In yet other embodiments, the assay is a single molecule detection assay. In yet other embodiments, the assay is an immunoassay, the subject is a human and the sample is whole blood. In yet other embodiments, the assay is a point-of-care assay, the subject is a human and the sample is whole blood. In yet other embodiments, the assay is a clinical chemistry assay and the sample is whole blood. In still further embodiments, the assay is a single molecule detection assay and the sample is whole blood. In yet other embodiments, the assay is an immunoassay, the subject is a human and the sample is serum. In yet other embodiments, the assay is a point-of-care assay, the subject is a human and the sample is serum. In yet other embodiments, the assay is a clinical chemistry assay and the sample is serum. In still further embodiments, the assay is a single molecule detection assay and the sample is serum. In yet other embodiments, the assay is an immunoassay, the subject is a human and the sample is plasma. In yet other embodiments, the assay is a point-of-care assay, the subject is a human and the sample is plasma. In yet other embodiments, the assay is a clinical chemistry assay and the sample is plasma. In still further embodiments, the assay is a single molecule detection assay and the sample is plasma.

[0020] In yet another embodiment, the present disclosure relates a method of aiding in the determination of whether to perform a head computerized tomography (CT) scan on a human subject that has sustained or may have sustained a suspected injury to the head. The method comprises: performing an assay on a sample obtained from the subject within about 2 hours after a suspected injury to the head to measure or detect a level of an early biomarker in the sample, said early biomarker comprising ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), glial fibrillary acidic protein (GFAP), or a combination thereof, in the sample; and performing a CT scan on the subject when the level of the early biomarker in the sample is higher than a reference level of the early biomarker and not performing a CT scan on the subject when the level of the early biomarker in the sample is lower than a reference level of the early biomarker. Depending on the clinical circumstances of a subject, the skilled person will select the appropriate early biomarker to be employed in the methods described herein. For example, in some instances based on the clinical circumstances of the subject, the skilled person will assay only for the biomarker UCH-L1. In other instances, based on the clinical circumstances, the skilled person will assay only for the biomarker GFAP. In other instances,

based on the clinical circumstances, the skilled person will assay for both the biomarkers UCH-L1 and GFAP. UCH-L1, GFAP, or the combination of UCH-L1 and GFAP may be assayed alone, or, may be combined with an assessment of one or more other biomarkers. In other aspects, the methods described herein may be performed more than once on a subject with the skilled person assaying for different early biomarkers and combinations thereof depending on the clinical circumstances of the subject.

[0021] In still yet another embodiment, the present disclosure relates a method of evaluating whether to perform a head computerized tomography (CT) scan on a human subject that has sustained or may have sustained a suspected injury to the head. The method comprises: performing an assay on a sample obtained from the subject within about 2 hours after a suspected injury to the head to measure a level of an early biomarker in the sample, said early biomarker comprising ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), glial fibrillary acidic protein (GFAP), or a combination thereof, in the sample; and performing a CT scan on the subject when the level of the early biomarker in the sample is higher than a reference level of the early biomarker and not performing a CT scan on the subject when the level of the early biomarker in the sample is lower than a reference level of the early biomarker. Depending on the clinical circumstances of a subject, the skilled person will select the appropriate early biomarker to be employed in the methods described herein. For example, in some instances based on the clinical circumstances of the subject, the skilled person will assay only for the biomarker UCH-L1. In other instances, based on the clinical circumstances, the skilled person will assay only for the biomarker GFAP. In other instances, based on the clinical circumstances, the skilled person will assay for both the biomarkers UCH-L1 and GFAP. UCH-L1, GFAP, or the combination of UCH-L1 and GFAP may be assayed alone, or, may be combined with an assessment of one or more other biomarkers. In other aspects, the methods described herein may be performed more than once on a subject with the skilled person assaying for different early biomarkers and combinations thereof depending on the clinical circumstances of the subject.

[0022] In the above-described methods for determining or evaluating whether to perform a head CT, the subject may be suspected of having a traumatic brain injury based on a CT scan that has been or already was performed (meaning, prior to the assay being performed). For example, depending upon a subject's medical condition (such as, if the patient is unconscious), a CT scan may be conducted shortly after the subject arrives at an emergency room, trauma center, or other site in order to assess and/or evaluate whether the subject has a TBI. Such a CT scan may be performed prior to the assay being performed to confirm and

determine whether or not the subject has a mild or moderate to severe TBI. After the assay is performed, one or more subsequent CT scans can be performed based on the results of the assay as part of the physician's (or other medical personnel's) management of the TBI (such as, for example, to determine whether surgical and/or pharmacological intervention may be required).

[0023] In certain aspects of the above methods, the subject may be suspected of having a traumatic brain injury based on a CT scan. For example, a subject may be suspected of having a mild TBI based on a CT scan. Alternatively, a subject may be suspected of having a moderate TBI based on a CT scan. Alternatively, a subject may be suspected of having a severe TBI based on a CT scan. Alternatively, a subject may be suspected of having a moderate to severe TBI based on a CT scan. Still further, a subject may be suspected of not having a TBI based on a CT scan.

[0024] In certain aspects of the above methods, the reference level used is correlated or corresponds to a positive head computed tomography. For example, the reference level can correlate or correspond (such as through an increase or decrease in the reference level) to subjects having a positive head computed tomography. Alternatively, the reference level can correlate or correspond (such as through an increase or decrease in the reference level) to subjects having negative head computed tomography. Still further alternatively, the reference level can correlate or correspond (such as through an increase or decrease in the reference level) to subjects experiencing a brain bleed or a brain bleed that is improving or getting worse. In other aspects of the above method, the reference level is correlated or corresponds to control subjects which have not suffered any TBI.

[0025] In the above-described methods, the reference level can comprise a recited value or range of values. For example, in one aspect, the reference level for GFAP can be about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL of a biological sample, such as, for example, a whole blood sample, a serum sample, or a plasma sample. In another aspect, the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL of a biological sample, such as, for example, a whole blood sample, a serum sample, or a plasma sample. In yet another aspect, the reference level for UCH-L1 is about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL of a biological sample, such as, for example a whole blood sample, a serum sample or a plasma sample. In yet still a further aspect, the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL of a biological sample, such as, for example, a whole blood

sample, a serum sample or a plasma sample. In another aspect, the reference level for GFAP can be about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL of a whole blood sample. In another aspect, the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL of a whole blood sample. In yet another aspect, the reference level for UCH-L1 is about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL of a a whole blood sample. In yet still a further aspect, the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL of a serum sample. In yet another aspect, the reference level for GFAP can be about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL of a serum sample. In another aspect, the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL of a serum sample. In yet another aspect, the reference level for UCH-L1 is about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL of a serum sample. In yet still a further aspect, the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL of a serum sample. In still yet a further aspect, the reference level is (a) determined by an assay having a sensitivity of between at least about 65% to 100% and a specificity of between at least about 30% to 100%; (b) determined by an assay having a sensitivity of at least about 99% and a specificity of at least about 75%; (c) between at least about 5 pg/mL to about 3500 pg/mL; or (d) between at least about 10 pg/mL to about 1000 pg/mL of a biological sample, such as, for example, a whole blood sample, a serum sample or a plasma sample. In still yet a further aspect, the reference level is (a) determined by an assay having a sensitivity of between at least about 65% to 100% and a specificity of between at least about 30% to 100%; (b) determined by an assay having a sensitivity of at least about 99% and a specificity of at least about 75%; (c) between at least about 5 pg/mL to about 3500 pg/mL; or (d) between at least about 10 pg/mL to about 1000 pg/mL of a whole blood sample. In still yet a further aspect, the reference level is (a) determined by an assay having a sensitivity of between at least about 65% to 100% and a specificity of between at least about 30% to 100%; (b) determined by an assay having a sensitivity of at least about 99% and a specificity of at least about 75%; (c) between at least about 5 pg/mL to about 3500 pg/mL; or (d) between at least about 10 pg/mL to about 1000 pg/mL of a serum sample. In still yet a further aspect, the reference level is (a) determined by an assay having a sensitivity of between at least about 65% to 100% and a specificity of between at least about 30% to 100%; (b) determined by an assay having a sensitivity of at least about 99% and a specificity

of at least about 75%; (c) between at least about 5 pg/mL to about 3500 pg/mL; or (d) between at least about 10 pg/mL to about 1000 pg/mL of a plasma sample. In still yet a further aspect, the reference level for UCH-L1 is from at least about 70 pg/mL to about 3500 pg/mL and the reference level for GFAP is from at least about 5 pg/mL to about 2000 pg/mL of a biological sample, such as, for example, a whole blood sample, a serum sample or a plasma sample. In still yet a further aspect, the reference level for UCH-L1 is from at least about 70 pg/mL to about 3500 pg/mL and the reference level for GFAP is from at least about 5 pg/mL to about 2000 pg/mL of a whole blood sample. In still yet a further aspect, the reference level for UCH-L1 is from at least about 70 pg/mL to about 3500 pg/mL and the reference level for GFAP is from at least about 5 pg/mL to about 2000 pg/mL of a serum sample. In still yet a further aspect, the reference level for UCH-L1 is from at least about 70 pg/mL to about 3500 pg/mL and the reference level for GFAP is from at least about 5 pg/mL to about 2000 pg/mL of a plasma sample.

[0026] In yet another aspect in the above-described methods, UCH-L1 can be measured by:

A. contacting the sample, either simultaneously or sequentially, in any order with:

(1) a UCH-L1-capture antibody, which binds to an epitope on UCH-L1 or UCH-L1 fragment to form a UCH-L1-capture antibody-UCH-L1 antigen complex, and

(2) a UCH-L1-detection antibody which includes a detectable label and binds to an epitope on UCH-L1 that is not bound by the UCH-L1-capture antibody, to form a UCH-L1 antigen-UCH-L1-detection antibody complex,

such that a UCH-L1-capture antibody-UCH-L1 antigen-UCH-L1-detection antibody complex is formed, and

B. measuring the amount or concentration of UCH-L1 in the sample based on the signal generated by the detectable label in the UCH-L1-capture antibody-UCH-L1 antigen-UCH-L1-detection antibody complex.

[0027] In yet another aspect in the above-described methods, GFAP can be measured by:

A. contacting the sample, either simultaneously or sequentially, in any order with:

(1) a GFAP-capture antibody, which binds to an epitope on GFAP or GFAP fragment to form a GFAP-capture antibody-GFAP antigen complex, and

(2) a GFAP-detection antibody which includes a detectable label and binds to an epitope on GFAP that is not bound by the GFAP-capture antibody, to form a GFAP antigen-GFAP-detection antibody complex,

such that a GFAP-capture antibody-GFAP antigen-GFAP-detection antibody complex is formed, and

B. measuring the amount or concentration of GFAP in the sample based on the signal generated by the detectable label in the GFAP-capture antibody-GFAP antigen-GFAP-detection antibody complex.

[0028] In the above-described methods, the sample can be taken within about 5 minutes after a suspected injury to the head. Alternatively, the sample can be taken within about 10 minutes of a suspected injury to the head. Alternatively, the sample can be taken within about 12 minutes of a suspected injury to the head. Alternatively, the sample can be taken within 15 minutes of a suspected injury to the head. Alternatively, the sample can be taken within about 20 minutes of a suspected injury to the head. Alternatively, the sample can be taken within 30 minutes of a suspected injury to the head. Alternatively, the sample can be taken within 60 minutes of a suspected injury to the head. Alternatively, the sample can be taken within 90 minutes of a suspected injury to the head.

[0029] The above-described methods can further comprise treating a human subject assessed or evaluated as having mild or moderate to severe TBI with a treatment for TBI (e.g., a surgical treatment, a therapeutic treatment, or combinations thereof). Any such treatment known in the art and described further herein can be used. Moreover, in a further aspect, any subject being treated for TBI can also, optionally, be monitored during and after any course of treatment. Alternatively, said methods can further comprise monitoring a subject assessed as having mild or moderate TBI (such as those, who as of yet, may not be receiving any treatment).

[0030] In the above-described methods, the sample can be selected from the group consisting of a whole blood sample, a serum sample, a cerebrospinal fluid sample, and a plasma sample. In some embodiments, the sample is a whole blood sample. In some embodiments, the sample is a plasma sample. In yet other embodiments, the sample is a serum sample. Such a sample can be obtained in a variety of ways. For example, the sample can be obtained after the subject sustained a head injury caused by physical shaking, blunt impact by an external mechanical or other force that results in a closed or open head trauma, one or more falls, explosions or blasts or other types of blunt force trauma. Alternatively, the sample can be obtained after the subject has ingested or been exposed to a chemical, toxin or

combination of a chemical and toxin. Examples of chemicals or toxins are fire, mold, asbestos, a pesticide, an insecticide, an organic solvent, a paint, a glue, a gas, an organic metal, a drug of abuse or one or more combinations thereof. Still further, the sample can be obtained from a subject that suffers from an autoimmune disease, a metabolic disorder, a brain tumor, hypoxia, a virus, meningitis, hydrocephalus or combinations thereof.

[0031] Any of the above-described methods can be carried out on any human subject without regard to factors selected from the group consisting of the human subject's clinical condition, the human subject's laboratory values, the human subject's classification as suffering from mild or moderate to severe TBI, the human subject's exhibition of low or high levels of UCH-L1, GFAP or UCH-L1 and GFAP and the timing of any event wherein the human subject may have sustained head injury.

[0032] In the above-described methods, the assay is an immunoassay. In some embodiments, the assay is a point-of-care assay. In yet other embodiments, the assay is a clinical chemistry assay. In yet other embodiments, the assay is a single molecule detection assay. In yet other embodiments, the assay is an immunoassay, the subject is a human and the sample is whole blood. In yet other embodiments, the assay is a point-of-care assay, the subject is a human and the sample is whole blood. In yet other embodiments, the assay is a clinical chemistry assay and the sample is whole blood. In still further embodiments, the assay is a single molecule detection assay and the sample is whole blood. In yet other embodiments, the assay is an immunoassay, the subject is a human and the sample is serum. In yet other embodiments, the assay is a point-of-care assay, the subject is a human and the sample is serum. In yet other embodiments, the assay is a clinical chemistry assay and the sample is serum. In still further embodiments, the assay is a single molecule detection assay and the sample is serum. In yet other embodiments, the assay is an immunoassay, the subject is a human and the sample is plasma. In yet other embodiments, the assay is a point-of-care assay, the subject is a human and the sample is plasma. In yet other embodiments, the assay is a clinical chemistry assay and the sample is plasma. In still further embodiments, the assay is a single molecule detection assay and the sample is plasma.

BRIEF DESCRIPTION OF THE DRAWINGS

[0033] FIG. 1 shows CT status (positive or negative CT scan results) and ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) levels of human subjects vs. blood draw time relative to injury.

[0034] FIG. 2 shows CT status and glial fibrillary acidic protein (GFAP) levels of human subjects vs. blood draw time relative to injury.

[0035] FIG. 3 shows Glasgow Coma Scale (GCS) Score result (mild, moderate, or severe TBI) and UCH-L1 levels of human subjects vs. blood draw time relative to injury.

[0036] FIG. 4 shows GCS Score result and GFAP levels of human subjects vs. blood draw time relative to injury.

[0037] FIG. 5 shows a box plot of UCH-L1 levels in human subjects with positive or negative CT scan results and UCH-L1 levels in control human subjects.

[0038] FIG. 6 shows a box plot of GFAP levels in human subjects with positive or negative CT scan results and GFAP levels in control human subjects.

[0039] FIG. 7 shows a receiver operating characteristic (ROC) analysis of UCH-L1 levels correlated with CT status (positive vs. negative CT scan result) in samples taken within about 2 hours of suspected injury.

[0040] FIG. 8 shows a receiver operating characteristic (ROC) analysis of GFAP levels correlated with CT status (positive vs. negative CT scan result) in samples taken within about 2 hours of suspected injury.

[0041] FIG. 9 shows a receiver operating characteristic (ROC) analysis of the combination of UCH-L1 levels and GFAP levels correlated with CT status (positive vs. negative CT scan result) in samples taken within about 2 hours of suspected injury.

[0042] FIG. 10 shows a box plot of UCH-L1 levels in human subjects having mild or moderate to severe TBI GCS scores compared with UCH-L1 levels in control human subjects.

[0043] FIG. 11 shows a box plot of GFAP levels in human subjects having mild or moderate to severe TBI GCS scores compared with GFAP levels in control human subjects.

[0044] FIG. 12 shows a receiver operating characteristic (ROC) analysis of UCH-L1 levels correlated with GCS Score result (mild vs. moderate/severe) in samples taken within about 2 hours of suspected injury.

[0045] FIG. 13 shows a receiver operating characteristic (ROC) analysis of GFAP levels correlated with GCS Score result (mild vs. moderate/severe) in samples taken within about 2 hours of suspected injury.

[0046] FIG. 14 shows a receiver operating characteristic (ROC) analysis of the combination of UCH-L1 levels and GFAP levels correlated with GCS Score result (mild vs. moderate/severe) in samples taken within about 2 hours of suspected injury.

DETAILED DESCRIPTION

[0047] The present invention relates to methods that aid in the hyperacute diagnosis and evaluation of a human subject that has sustained an injury to the head, such as mild, moderate, severe, or moderate to severe traumatic brain injury (TBI), using an early biomarker, such as ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), glial fibrillary acidic protein (GFAP), or a combination thereof. These methods involve detecting one or more early biomarker levels in one or more samples taken from the human subject at a time point within about 2 hours of the injury to the head or suspected injury to the head. The detection of levels of the early biomarker, such as UCH-L1, GFAP, or combination thereof, that are higher than reference levels of the early biomarker within about the first 2 hours after injury or suspected injury to the head provides an aid in accurately evaluating or diagnosing the human subject, thus allowing for the subsequent early treatment and monitoring of patients with mild, moderate, severe, or moderate to severe traumatic brain injury. For example, human subjects having levels of early biomarker, such as UCH-L1, GFAP, or combination thereof, higher than a reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, may also be identified as having moderate to severe traumatic brain injury.

[0048] The present invention also relates to methods that aid in the hyperacute determination of whether a human subject that has sustained an injury to the head would benefit from and thus receive a head computerized tomography (CT) scan based on the levels of an early biomarker, such as UCH-L1, GFAP, or combination thereof. These methods involve detecting levels of the early biomarker, such as UCH-L1, GFAP, or combination thereof, in one or more samples taken from the human subject at a time point within about 2 hours of the injury to the head or suspected injury to the head. The detection levels of the early biomarker, such as UCH-L1, GFAP, or combination thereof, that are higher than reference levels of the early biomarker, within about the first 2 hours after injury or suspected injury to the head provides an aid in the hyperacute determination of whether a human subject should receive a head CT scan. For example, human subjects having a level of the early biomarker, such as UCH-L1, GFAP, or combination thereof, higher than a reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, may also be identified as likely to have a positive head CT scan and thus benefit from having a CT scan.

[0049] Section headings as used in this section and the entire disclosure herein are merely for organizational purposes and are not intended to be limiting.

1. Definitions

[0050] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. In case of conflict, the present document, including definitions, will control. Preferred methods and materials are described below, although methods and materials similar or equivalent to those described herein can be used in practice or testing of the present invention. All publications, patent applications, patents and other references mentioned herein are incorporated by reference in their entirety. The materials, methods, and examples disclosed herein are illustrative only and not intended to be limiting.

[0051] The terms "comprise(s)," "include(s)," "having," "has," "can," "contain(s)," and variants thereof, as used herein, are intended to be open-ended transitional phrases, terms, or words that do not preclude the possibility of additional acts or structures. The singular forms "a," "and" and "the" include plural references unless the context clearly dictates otherwise. The present disclosure also contemplates other embodiments "comprising," "consisting of" and "consisting essentially of," the embodiments or elements presented herein, whether explicitly set forth or not.

[0052] For the recitation of numeric ranges herein, each intervening number there between with the same degree of precision is explicitly contemplated. For example, for the range of 6-9, the numbers 7 and 8 are contemplated in addition to 6 and 9, and for the range 6.0-7.0, the number 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6, 6.7, 6.8, 6.9, and 7.0 are explicitly contemplated.

[0053] "Affinity matured antibody" is used herein to refer to an antibody with one or more alterations in one or more CDRs, which result in an improvement in the affinity (*i.e.*, K_D, k_d or k_a) of the antibody for a target antigen compared to a parent antibody, which does not possess the alteration(s). Exemplary affinity matured antibodies will have nanomolar or even picomolar affinities for the target antigen. A variety of procedures for producing affinity matured antibodies is known in the art, including the screening of a combinatory antibody library that has been prepared using bio-display. For example, Marks *et al.*, *BioTechnology*, 10: 779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by Barbas *et al.*, *Proc. Nat. Acad. Sci. USA*, 91: 3809-3813 (1994); Schier *et al.*, *Gene*, 169: 147-155 (1995); Yelton *et al.*, *J. Immunol.*, 155: 1994-2004 (1995); Jackson *et al.*, *J. Immunol.*, 154(7): 3310-3319 (1995); and Hawkins *et al*, J. Mol. Biol., 226: 889-896 (1992). Selective mutation at

selective mutagenesis positions and at contact or hypermutation positions with an activity-enhancing amino acid residue is described in U.S. Patent No. 6,914,128 B1.

[0054] "Antibody" and "antibodies" as used herein refers to monoclonal antibodies, multispecific antibodies, human antibodies, humanized antibodies (fully or partially humanized), animal antibodies such as, but not limited to, a bird (for example, a duck or a goose), a shark, a whale, and a mammal, including a non-primate (for example, a cow, a pig, a camel, a llama, a horse, a goat, a rabbit, a sheep, a hamster, a guinea pig, a cat, a dog, a rat, a mouse, etc.) or a non-human primate (for example, a monkey, a chimpanzee, etc.), recombinant antibodies, chimeric antibodies, single-chain Fvs ("scFv"), single chain antibodies, single domain antibodies, Fab fragments, F(ab') fragments, $F(ab')_2$ fragments, disulfide-linked Fvs ("sdFv"), and anti-idiotypic ("anti-Id") antibodies, dual-domain antibodies, dual variable domain (DVD) or triple variable domain (TVD) antibodies (dual-variable domain immunoglobulins and methods for making them are described in Wu, C., *et al.*, *Nature Biotechnology*, 25(11):1290-1297 (2007) and PCT International Application WO 2001/058956, the contents of each of which are herein incorporated by reference), and functionally active epitope-binding fragments of any of the above. In particular, antibodies include immunoglobulin molecules and immunologically active fragments of immunoglobulin molecules, namely, molecules that contain an analyte-binding site. Immunoglobulin molecules can be of any type (for example, IgG, IgE, IgM, IgD, IgA, and IgY), class (for example, IgG1, IgG2, IgG3, IgG4, IgA1, and IgA2), or subclass. For simplicity sake, an antibody against an analyte is frequently referred to herein as being either an "anti-analyte antibody" or merely an "analyte antibody" (*e.g.*, an anti-UCH-L1 antibody or a UCH-L1 antibody).

[0055] "Antibody fragment" as used herein refers to a portion of an intact antibody comprising the antigen-binding site or variable region. The portion does not include the constant heavy chain domains (*i.e.*, CH2, CH3, or CH4, depending on the antibody isotype) of the Fc region of the intact antibody. Examples of antibody fragments include, but are not limited to, Fab fragments, Fab' fragments, Fab'-SH fragments, $F(ab')_2$ fragments, Fd fragments, Fv fragments, diabodies, single-chain Fv (scFv) molecules, single-chain polypeptides containing only one light chain variable domain, single-chain polypeptides containing the three CDRs of the light-chain variable domain, single-chain polypeptides containing only one heavy chain variable region, and single-chain polypeptides containing the three CDRs of the heavy chain variable region.

[0056] The "area under curve" or "AUC" refers to area under a ROC curve. AUC under a ROC curve is a measure of accuracy. An AUC of 1 represents a perfect test, whereas an AUC of 0.5 represents an insignificant test. A preferred AUC may be at least approximately 0.700, at least approximately 0.750, at least approximately 0.800, at least approximately 0.850, at least approximately 0.900, at least approximately 0.910, at least approximately 0.920, at least approximately 0.930, at least approximately 0.940, at least approximately 0.950, at least approximately 0.960, at least approximately 0.970, at least approximately 0.980, at least approximately 0.990, or at least approximately 0.995.

[0057] "Bead" and "particle" are used herein interchangeably and refer to a substantially spherical solid support. One example of a bead or particle is a microparticle. Microparticles that can be used herein can be any type known in the art. For example, the bead or particle can be a magnetic bead or magnetic particle. Magnetic beads/particles may be ferromagnetic, ferrimagnetic, paramagnetic, superparamagnetic or ferrofluidic. Exemplary ferromagnetic materials include Fe, Co, Ni, Gd, Dy, CrO_2, MnAs, MnBi, EuO, and NiO/Fe. Examples of ferrimagnetic materials include $NiFe_2O_4$, $CoFe_2O_4$, Fe_3O_4 (or $FeO{\cdot}Fe_2O_3$). Beads can have a solid core portion that is magnetic and is surrounded by one or more non-magnetic layers. Alternately, the magnetic portion can be a layer around a non-magnetic core. The microparticles can be of any size that would work in the methods described herein, *e.g.*, from about 0.75 to about 5 nm, or from about 1 to about 5 nm, or from about 1 to about 3 nm.

[0058] "Binding protein" is used herein to refer to a monomeric or multimeric protein that binds to and forms a complex with a binding partner, such as, for example, a polypeptide, an antigen, a chemical compound or other molecule, or a substrate of any kind. A binding protein specifically binds a binding partner. Binding proteins include antibodies, as well as antigen-binding fragments thereof and other various forms and derivatives thereof as are known in the art and described herein below, and other molecules comprising one or more antigen-binding domains that bind to an antigen molecule or a particular site (epitope) on the antigen molecule. Accordingly, a binding protein includes, but is not limited to, an antibody a tetrameric immunoglobulin, an IgG molecule, an IgG1 molecule, a monoclonal antibody, a chimeric antibody, a CDR-grafted antibody, a humanized antibody, an affinity matured antibody, and fragments of any such antibodies that retain the ability to bind to an antigen.

[0059] "Bispecific antibody" is used herein to refer to a full-length antibody that is generated by quadroma technology (see Milstein *et al.*, *Nature*, 305(5934): 537-540 (1983)), by chemical conjugation of two different monoclonal antibodies (see, Staerz *et al.*, *Nature*, 314(6012): 628-631 (1985)), or by knob-into-hole or similar approaches, which introduce

mutations in the Fc region (see Holliger *et al.*, *Proc. Natl. Acad. Sci. USA*, 90(14): 6444-6448 (1993)), resulting in multiple different immunoglobulin species of which only one is the functional bispecific antibody. A bispecific antibody binds one antigen (or epitope) on one of its two binding arms (one pair of HC/LC), and binds a different antigen (or epitope) on its second arm (a different pair of HC/LC). By this definition, a bispecific antibody has two distinct antigen-binding arms (in both specificity and CDR sequences), and is monovalent for each antigen to which it binds to.

[0060] "CDR" is used herein to refer to the "complementarity determining region" within about an antibody variable sequence. There are three CDRs in each of the variable regions of the heavy chain and the light chain. Proceeding from the N-terminus of a heavy or light chain, these regions are denoted "CDR1", "CDR2", and "CDR3", for each of the variable regions. The term "CDR set" as used herein refers to a group of three CDRs that occur in a single variable region that binds the antigen. An antigen-binding site, therefore, may include six CDRs, comprising the CDR set from each of a heavy and a light chain variable region. A polypeptide comprising a single CDR, (*e.g.*, a CDR1, CDR2, or CDR3) may be referred to as a "molecular recognition unit." Crystallographic analyses of antigen-antibody complexes have demonstrated that the amino acid residues of CDRs form extensive contact with bound antigen, wherein the most extensive antigen contact is with the heavy chain CDR3. Thus, the molecular recognition units may be primarily responsible for the specificity of an antigen-binding site. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

[0061] The exact boundaries of these CDRs have been defined differently according to different systems. The system described by Kabat (Kabat *et al.*, *Sequences of Proteins of Immunological Interest* (National Institutes of Health, Bethesda, Md. (1987) and (1991)) not only provides an unambiguous residue numbering system applicable to any variable region of an antibody, but also provides precise residue boundaries defining the three CDRs. These CDRs may be referred to as "Kabat CDRs". Chothia and coworkers (Chothia and Lesk, *J. Mol. Biol.*, 196: 901-917 (1987); and Chothia *et al.*, *Nature*, 342: 877-883 (1989)) found that certain sub-portions within Kabat CDRs adopt nearly identical peptide backbone conformations, despite having great diversity at the level of amino acid sequence. These sub-portions were designated as "L1", "L2", and "L3", or "H1", "H2", and "H3", where the "L" and the "H" designate the light chain and the heavy chain regions, respectively. These regions may be referred to as "Chothia CDRs", which have boundaries that overlap with Kabat CDRs. Other boundaries defining CDRs overlapping with the Kabat CDRs have been

described by Padlan, *FASEB J.*, 9: 133-139 (1995), and MacCallum, *J. Mol. Biol.*, 262(5): 732-745 (1996). Still other CDR boundary definitions may not strictly follow one of the herein systems, but will nonetheless overlap with the Kabat CDRs, although they may be shortened or lengthened in light of prediction or experimental findings that particular residues or groups of residues or even entire CDRs do not significantly impact antigen binding. The methods used herein may utilize CDRs defined according to any of these systems, although certain embodiments use Kabat- or Chothia-defined CDRs.

[0062] "Component," "components," or "at least one component," refer generally to a capture antibody, a detection or conjugate a calibrator, a control, a sensitivity panel, a container, a buffer, a diluent, a salt, an enzyme, a co-factor for an enzyme, a detection reagent, a pretreatment reagent/solution, a substrate (*e.g.*, as a solution), a stop solution, and the like that can be included in a kit for assay of a test sample, such as a patient urine, whole blood, serum or plasma sample, in accordance with the methods described herein and other methods known in the art. Some components can be in solution or lyophilized for reconstitution for use in an assay.

[0063] "Correlated to" as used herein refers to compared to.

[0064] "CT scan" as used herein refers to a computerized tomography (CT) scan. A CT scan combines a series of X-ray images taken from different angles and uses computer processing to create cross-sectional images, or slices, of the bones, blood vessels and soft tissues inside your body. The CT scan may use X-ray CT, positron emission tomography (PET), single-photon emission computed tomography (SPECT), computed axial tomography (CAT scan), or computer aided tomography. The CT scan may be a conventional CT scan or a spiral/helical CT scan. In a conventional CT scan, the scan is taken slice by slice and after each slice the scan stops and moves down to the next slice, *e.g.*, from the top of the abdomen down to the pelvis. The conventional CT scan requires patients to hold their breath to avoid movement artefact. The spiral/helical CT scan is a continuous scan which is taken in a spiral fashion and is a much quicker process where the scanned images are contiguous.

[0065] "Derivative" of an antibody as used herein may refer to an antibody having one or more modifications to its amino acid sequence when compared to a genuine or parent antibody and exhibit a modified domain structure. The derivative may still be able to adopt the typical domain configuration found in native antibodies, as well as an amino acid sequence, which is able to bind to targets (antigens) with specificity. Typical examples of antibody derivatives are antibodies coupled to other polypeptides, rearranged antibody domains, or fragments of antibodies. The derivative may also comprise at least one further

compound, *e.g.*, a protein domain, said protein domain being linked by covalent or non-covalent bonds. The linkage can be based on genetic fusion according to the methods known in the art. The additional domain present in the fusion protein comprising the antibody may preferably be linked by a flexible linker, advantageously a peptide linker, wherein said peptide linker comprises plural, hydrophilic, peptide-bonded amino acids of a length sufficient to span the distance between the C-terminal end of the further protein domain and the N-terminal end of the antibody or vice versa. The antibody may be linked to an effector molecule having a conformation suitable for biological activity or selective binding to a solid support, a biologically active substance (*e.g.*, a cytokine or growth hormone), a chemical agent, a peptide, a protein, or a drug, for example.

[0066] "Determined by an assay" is used herein to refer to the determination of a reference level by any appropriate assay. The determination of a reference level may, in some embodiments, be achieved by an assay of the same type as the assay that is to be applied to the sample from the subject (for example, by an immunoassay, clinical chemistry assay, a single molecule detection assay, protein immunoprecipitation, immunoelectrophoresis, chemical analysis, SDS-PAGE and Western blot analysis, or protein immunostaining, electrophoresis analysis, a protein assay, a competitive binding assay, a functional protein assay, or chromatography or spectrometry methods, such as high-performance liquid chromatography (HPLC) or liquid chromatography–mass spectrometry (LC/MS)). The determination of a reference level may, in some embodiments, be achieved by an assay of the same type and under the same assay conditions as the assay that is to be applied to the sample from the subject. As noted herein, this disclosure provides exemplary reference levels (e.g., calculated by comparing reference levels at different time points). It is well within the ordinary skill of one in the art to adapt the disclosure herein for other assays to obtain assay-specific reference levels for those other assays based on the description provided by this disclosure. For example, a set of training samples comprising samples obtained from human subjects known to have sustained an injury to the head (and more particularly, samples obtained from human subjects known to have sustained a (i) mild TBI; and/or (ii) moderate, severe, or moderate to severe TBI and samples obtained from human subjects known not to have sustained an injury to the head may be used to obtain assay-specific reference levels. It will be understood that a reference level "determined by an assay" and having a recited level of "sensitivity" and/or "specificity" is used herein to refer to a reference level which has been determined to provide a method of the recited sensitivity and/or specificity when said reference level is adopted in the methods of the invention. It is well within the ordinary skill

of one in the art to determine the sensitivity and specificity associated with a given reference level in the methods of the invention, for example by repeated statistical analysis of assay data using a plurality of different possible reference levels.

[0067] Practically, when discriminating between a subject as having a traumatic brain injury or not having a traumatic brain injury or a subject as having a a mild versus a moderate, severe, or moderate to severetraumatic brain injury, the skilled person will balance the effect of raising a cutoff on sensitivity and specificity. Raising or lowering a cutoff will have a well-defined and predictable impact on sensitivity and specificity, and other standard statistical measures. It is well known that raising a cutoff will improve specificity but is likely to worsen sensitivity (proportion of those with disease who test positive). In contrast, lowering a cutoff will improve sensitivity but will worsen specificity (proportion of those without disease who test negative). The ramifications for detecting traumatic brain injury or determining a mild versus moderate, severe, or moderate to severe traumatic brain injury will be readily apparent to those skilled in the art. In discriminating whether a subject has or does not have a traumatic brain injury or a mild versus a moderate, severe, or moderate to severe traumatic brain injury, the higher the cutoff, specificity improves as more true negatives (i.e., subjects not having a traumatic brain injury, not having a mild traumatic brain injury, not have a moderate traumatic brain injury, not having a severe traumatic brain injury or not having a moderate to severe traumatic brain injury) are distinguished from those having a traumatic brain injury, a mild traumatic brain injury, a moderate traumatic brain injury, a severe traumatic brain injury or a moderate to severe traumatic brain injury. But at the same time, raising the cutoff decreases the number of cases identified as positive overall, as well as the number of true positives, so the sensitivity must decrease. Conversely, the lower the cutoff, sensitivity improves as more true positives (i.e., subjects having a traumatic brain injury, having a mild traumatic brain injury, having a moderate traumatic brain injury, having a severe traumatic brain injury or having a moderate to severe traumatic brain injury) are distinguished from those who do not have a traumatic brain injury, a mild traumatic brain injure, a moderate traumatic brain injury, a severe traumatic brain injury or a moderate to severe traumatic brain injury. But at the same time, lowering the cutoff increases the number of cases identified as positive overall, as well as the number of false positives, so the specificity must decrease.

[0068] Generally, a high sensitivity value helps one of skill rule out disease or condition (such as a traumatic brain injury, mild traumatic brain injury, moderate traumatic brain injury, severe traumatic brain injury or moderate to severe traumatic brain injury), and a high

specificity value helps one of skill rule in disease or condition. Whether one of skill desires to rule out or rule in disease depends on what the consequences are for the patient for each type of error. Accordingly, one cannot know or predict the precise balancing employed to derive a test cutoff without full disclosure of the underlying information on how the value was selected. The balancing of sensitivity against specificity and other factors will differ on a case-by-case basis. This is why it is sometimes preferable to provide alternate cutoff (e.g., reference) values so a physican or practitioner can choose.

[0069] "Drugs of abuse" is used herein to refer to one or more additive substances (such as a drug) taken for non-medical reasons (such as for, example, recreational and/or mind-altering effects). Excessive overindulgence, use or dependence of such drugs of abuse is often referred to as "substance abuse". Examples of drugs of abuse include alcohol, barbiturates, benzodiazepines, cannabis, cocaine, hallucinogens (such as ketamine, mescaline (peyote), PCP, psilocybin, DMT and/or LSD), methaqualone, opioids, amphetamines (including methamphetamines), anabolic steroids, inhalants (namely, substances which contain volatile substances that contain psychoactive properties such as, for example, nitrites, spray paints, cleaning fluids, markers, glues, etc.) and combinations thereof.

[0070] "Dual-specific antibody" is used herein to refer to a full-length antibody that can bind two different antigens (or epitopes) in each of its two binding arms (a pair of HC/LC) (see PCT publication WO 02/02773). Accordingly, a dual-specific binding protein has two identical antigen binding arms, with identical specificity and identical CDR sequences, and is bivalent for each antigen to which it binds.

[0071] "Dual variable domain" is used herein to refer to two or more antigen binding sites on a binding protein, which may be divalent (two antigen binding sites), tetravalent (four antigen binding sites), or multivalent binding proteins. DVDs may be monospecific, *i.e.*, capable of binding one antigen (or one specific epitope), or multispecific, *i.e.*, capable of binding two or more antigens (*i.e.*, two or more epitopes of the same target antigen molecule or two or more epitopes of different target antigens). A preferred DVD binding protein comprises two heavy chain DVD polypeptides and two light chain DVD polypeptides and is referred to as a "DVD immunoglobulin" or "DVD-Ig." Such a DVD-Ig binding protein is thus tetrameric and reminiscent of an IgG molecule, but provides more antigen binding sites than an IgG molecule. Thus, each half of a tetrameric DVD-Ig molecule is reminiscent of one half of an IgG molecule and comprises a heavy chain DVD polypeptide and a light chain DVD polypeptide, but unlike a pair of heavy and light chains of an IgG molecule that

provides a single antigen binding domain, a pair of heavy and light chains of a DVD-Ig provide two or more antigen binding sites.

[0072] Each antigen binding site of a DVD-Ig binding protein may be derived from a donor ("parental") monoclonal antibody and thus comprises a heavy chain variable domain (VH) and a light chain variable domain (VL) with a total of six CDRs involved in antigen binding per antigen binding site. Accordingly, a DVD-Ig binding protein that binds two different epitopes (*i.e.*, two different epitopes of two different antigen molecules or two different epitopes of the same antigen molecule) comprises an antigen binding site derived from a first parental monoclonal antibody and an antigen binding site of a second parental monoclonal antibody.

[0073] A description of the design, expression, and characterization of DVD-Ig binding molecules is provided in PCT Publication No. WO 2007/024715, U.S. Patent No. 7,612,181, and Wu *et al.*, *Nature Biotech.*, 25: 1290-1297 (2007). A preferred example of such DVD-Ig molecules comprises a heavy chain that comprises the structural formula VD1-(X1)n-VD2-C-(X2)n, wherein VD1 is a first heavy chain variable domain, VD2 is a second heavy chain variable domain, C is a heavy chain constant domain, X1 is a linker with the proviso that it is not CH1, X2 is an Fc region, and n is 0 or 1, but preferably 1; and a light chain that comprises the structural formula VD1-(X1)n-VD2-C-(X2)n, wherein VD1 is a first light chain variable domain, VD2 is a second light chain variable domain, C is a light chain constant domain, X1 is a linker with the proviso that it is not CH1, and X2 does not comprise an Fc region; and n is 0 or 1, but preferably 1. Such a DVD-Ig may comprise two such heavy chains and two such light chains, wherein each chain comprises variable domains linked in tandem without an intervening constant region between variable regions, wherein a heavy chain and a light chain associate to form tandem functional antigen binding sites, and a pair of heavy and light chains may associate with another pair of heavy and light chains to form a tetrameric binding protein with four functional antigen binding sites. In another example, a DVD-Ig molecule may comprise heavy and light chains that each comprise three variable domains (VD1, VD2, VD3) linked in tandem without an intervening constant region between variable domains, wherein a pair of heavy and light chains may associate to form three antigen binding sites, and wherein a pair of heavy and light chains may associate with another pair of heavy and light chains to form a tetrameric binding protein with six antigen binding sites.

[0074] In a preferred embodiment, a DVD-Ig binding protein not only binds the same target molecules bound by its parental monoclonal antibodies, but also possesses one or more desirable properties of one or more of its parental monoclonal antibodies. Preferably, such an

additional property is an antibody parameter of one or more of the parental monoclonal antibodies. Antibody parameters that may be contributed to a DVD-Ig binding protein from one or more of its parental monoclonal antibodies include, but are not limited to, antigen specificity, antigen affinity, potency, biological function, epitope recognition, protein stability, protein solubility, production efficiency, immunogenicity, pharmacokinetics, bioavailability, tissue cross reactivity, and orthologous antigen binding.

[0075] A DVD-Ig binding protein binds at least one epitope of UCH-L1. Non-limiting examples of a DVD-Ig binding protein include a DVD-Ig binding protein that binds one or more epitopes of UCH-L1, a DVD-Ig binding protein that binds an epitope of a human UCH-L1 and an epitope of UCH-L1 of another species (for example, mouse), and a DVD-Ig binding protein that binds an epitope of a human UCH-L1 and an epitope of another target molecule.

[0076] "Dynamic range" as used herein refers to range over which an assay readout is proportional to the amount of target molecule or analyte in the sample being analyzed.

[0077] "Epitope," or "epitopes," or "epitopes of interest" refer to a site(s) on any molecule that is recognized and can bind to a complementary site(s) on its specific binding partner. The molecule and specific binding partner are part of a specific binding pair. For example, an epitope can be on a polypeptide, a protein, a hapten, a carbohydrate antigen (such as, but not limited to, glycolipids, glycoproteins or lipopolysaccharides), or a polysaccharide. Its specific binding partner can be, but is not limited to, an antibody.

[0078] "Fragment antigen-binding fragment" or "Fab fragment" as used herein refers to a fragment of an antibody that binds to antigens and that contains one antigen-binding site, one complete light chain, and part of one heavy chain. Fab is a monovalent fragment consisting of the VL, VH, CL and CH1 domains. Fab is composed of one constant and one variable domain of each of the heavy and the light chain. The variable domain contains the paratope (the antigen-binding site), comprising a set of complementarity determining regions, at the amino terminal end of the monomer. Each arm of the Y thus binds an epitope on the antigen. Fab fragments can be generated such as has been described in the art, e.g., using the enzyme papain, which can be used to cleave an immunoglobulin monomer into two Fab fragments and an Fc fragment, or can be produced by recombinant means.

[0079] "$F(ab')_2$ fragment" as used herein refers to antibodies generated by pepsin digestion of whole IgG antibodies to remove most of the Fc region while leaving intact some of the hinge region. $F(ab')_2$ fragments have two antigen-binding F(ab) portions linked together by disulfide bonds, and therefore are divalent with a molecular weight of about 110 kDa.

Divalent antibody fragments ($F(ab')_2$ fragments) are smaller than whole IgG molecules and enable a better penetration into tissue thus facilitating better antigen recognition in immunohistochemistry. The use of $F(ab')_2$ fragments also avoids unspecific binding to Fc receptor on live cells or to Protein A/G. $F(ab')_2$ fragments can both bind and precipitate antigens.

[0080] "Framework" (FR) or "Framework sequence" as used herein may mean the remaining sequences of a variable region minus the CDRs. Because the exact definition of a CDR sequence can be determined by different systems (for example, see above), the meaning of a framework sequence is subject to correspondingly different interpretations. The six CDRs (CDR-L1, -L2, and -L3 of light chain and CDR-H1, -H2, and -H3 of heavy chain) also divide the framework regions on the light chain and the heavy chain into four sub-regions (FR1, FR2, FR3, and FR4) on each chain, in which CDR1 is positioned between FR1 and FR2, CDR2 between FR2 and FR3, and CDR3 between FR3 and FR4. Without specifying the particular sub-regions as FR1, FR2, FR3, or FR4, a framework region, as referred by others, represents the combined FRs within the variable region of a single, naturally occurring immunoglobulin chain. As used herein, a FR represents one of the four sub-regions, and FRs represents two or more of the four sub-regions constituting a framework region.

[0081] Human heavy chain and light chain FR sequences are known in the art that can be used as heavy chain and light chain "acceptor" framework sequences (or simply, "acceptor" sequences) to humanize a non-human antibody using techniques known in the art. In one embodiment, human heavy chain and light chain acceptor sequences are selected from the framework sequences listed in publicly available databases such as V-base (hypertext transfer protocol://vbase.mrc-cpe.cam.ac.uk/) or in the international ImMunoGeneTics® (IMGT®) information system (hypertext transfer protocol://imgt.cines.fr/texts/IMGTrepertoire/LocusGenes/).

[0082] "Functional antigen binding site" as used herein may mean a site on a binding protein (*e.g.*, an antibody) that is capable of binding a target antigen. The antigen binding affinity of the antigen binding site may not be as strong as the parent binding protein, *e.g.*, parent antibody, from which the antigen binding site is derived, but the ability to bind antigen must be measurable using any one of a variety of methods known for evaluating protein, *e.g.*, antibody, binding to an antigen. Moreover, the antigen binding affinity of each of the antigen binding sites of a multivalent protein, *e.g.*, multivalent antibody, herein need not be quantitatively the same.

[0083] "GFAP" is used herein to describe glial fibrillary acidic protein. GFAP is a protein that is encoded by the *GFAP* gene in humans, and which can be produced (e.g., by recombinant means, in other species).

[0084] "GFAP status" can mean either the level or amount of GFAP at a point in time (such as with a single measure of GFAP), the level or amount of GFAP associated with monitoring (such as with a repeat test on a subject to identify an increase or decrease in GFAP amount), the level or amount of GFAP associated with treatment for traumatic brain injury (whether a primary brain injury and/or a secondary brain injury) or combinations thereof.

[0085] "Glasgow Coma Scale" or "GCS" as used herein refers to a 15 point scale for estimating and categorizing the outcomes of brain injury on the basis of overall social capability or dependence on others. The test measures the motor response, verbal response and eye opening response with these values: I. Motor Response (6 – Obeys commands fully; 5 – Localizes to noxious stimuli; 4 – Withdraws from noxious stimuli; 3 – Abnormal flexion, *i.e.*, decorticate posturing; 2 – Extensor response, *i.e.*, decerebrate posturing; and 1 – No response); II. Verbal Response (5 – Alert and Oriented; 4 – Confused, yet coherent, speech; 3 – Inappropriate words and jumbled phrases consisting of words; 2 – Incomprehensible sounds; and 1 – No sounds); and III. Eye Opening (4 – Spontaneous eye opening; 3 – Eyes open to speech; 2 – Eyes open to pain; and 1 – No eye opening). The final score is determined by adding the values of I+II+III. The final score can be categorized into four possible levels for survival, with a lower number indicating a more severe injury and a poorer prognosis: Mild (13-15); Moderate Disability (9-12) (Loss of consciousness greater than 30 minutes; Physical or cognitive impairments which may or may resolve: and Benefit from Rehabilitation); Severe Disability (3-8) (Coma: unconscious state. No meaningful response, no voluntary activities); and Vegetative State (Less Than 3) (Sleep wake cycles; Arousal, but no interaction with environment; No localized response to pain). Moderate brain injury is defined as a brain injury resulting in a loss of consciousness from 20 minutes to 6 hours and a Glasgow Coma Scale of 9 to 12. Severe brain injury is defined as a brain injury resulting in a loss of consciousness of greater than 6 hours and a Glasgow Coma Scale of 3 to 8.

[0086] "Glasgow Outcome Scale" as used herein refers to a global scale for functional outcome that rates patient status into one of five categories: Dead, Vegetative State, Severe Disability, Moderate Disability or Good Recovery.

[0087] "Extended Glasgow Outcome Scale" or "GOSE" as used interchangeably herein provides more detailed categorization into eight categories by subdividing the categories of

severe disability, moderate disability and good recovery into a lower and upper category as shown in Table 1.

Table 1

1	Death	D	
2	Vegetative state	VX	
3	Lower severe disability	SD -	Condition of unawareness with only reflex responses but with periods of spontaneous eye opening
4	Upper severe disability	SD +	
5	Lower moderate disability	MD -	Patient who is dependent for daily support for mental or physical disability, usually a combination of both. If the patient can be left alone for more than 8 hours at home it is upper level of SD, if not then it is low level of SD.
6	Upper moderate disability	MD +	
7	Lower good recovery	GR -	Patients have some disability such as aphasia, hemiparesis or epilepsy and/or deficits of memory or personality but are able to look after themselves. They are independent at home but dependent outside. If they are able to return to work even with special arrangement it is upper level of MD, if not then it is low level of MD.
8	Upper good recovery	GR +	

[0088] "Humanized antibody" is used herein to describe an antibody that comprises heavy and light chain variable region sequences from a non-human species (*e.g.*, a mouse) but in which at least a portion of the VH and/or VL sequence has been altered to be more "human-like," *i.e.*, more similar to human germline variable sequences. A "humanized antibody" is an antibody or a variant, derivative, analog, or fragment thereof, which immunospecifically binds to an antigen of interest and which comprises a framework (FR) region having substantially the amino acid sequence of a human antibody and a complementary determining region (CDR) having substantially the amino acid sequence of a non-human antibody. As used herein, the term "substantially" in the context of a CDR refers to a CDR having an amino acid sequence at least 80%, at least 85%, at least 90%, at least 95%, at least 98%, or at least 99% identical to the amino acid sequence of a non-human antibody CDR. A humanized antibody comprises substantially all of at least one, and typically two, variable domains (Fab, Fab', $F(ab')_2$, FabC, Fv) in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin (*i.e.*, donor antibody) and all or substantially all of the framework regions are those of a human immunoglobulin consensus sequence. In an embodiment, a humanized antibody also comprises at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. In some embodiments, a

humanized antibody contains the light chain as well as at least the variable domain of a heavy chain. The antibody also may include the CH1, hinge, CH2, CH3, and CH4 regions of the heavy chain. In some embodiments, a humanized antibody only contains a humanized light chain. In some embodiments, a humanized antibody only contains a humanized heavy chain. In specific embodiments, a humanized antibody only contains a humanized variable domain of a light chain and/or humanized heavy chain.

[0089] A humanized antibody can be selected from any class of immunoglobulins, including IgM, IgG, IgD, IgA, and IgE, and any isotype, including without limitation IgG1, IgG2, IgG3, and IgG4. A humanized antibody may comprise sequences from more than one class or isotype, and particular constant domains may be selected to optimize desired effector functions using techniques well-known in the art.

[0090] The framework regions and CDRs of a humanized antibody need not correspond precisely to the parental sequences, *e.g.*, the donor antibody CDR or the consensus framework may be mutagenized by substitution, insertion, and/or deletion of at least one amino acid residue so that the CDR or framework residue at that site does not correspond to either the donor antibody or the consensus framework. In a preferred embodiment, such mutations, however, will not be extensive. Usually, at least 80%, preferably at least 85%, more preferably at least 90%, and most preferably at least 95% of the humanized antibody residues will correspond to those of the parental FR and CDR sequences. As used herein, the term "consensus framework" refers to the framework region in the consensus immunoglobulin sequence. As used herein, the term "consensus immunoglobulin sequence" refers to the sequence formed from the most frequently occurring amino acids (or nucleotides) in a family of related immunoglobulin sequences (see, *e.g.*, Winnaker, *From Genes to Clones* (Verlagsgesellschaft, Weinheim, 1987)). A "consensus immunoglobulin sequence" may thus comprise a "consensus framework region(s)" and/or a "consensus CDR(s)". In a family of immunoglobulins, each position in the consensus sequence is occupied by the amino acid occurring most frequently at that position in the family. If two amino acids occur equally frequently, either can be included in the consensus sequence.

[0091] "Hyperacute" as used herein refers to extremely acute or within a course of about 2 hours of the injury or suspected injury to the head. Hyperacute is within an early stage, e.g., a hyperacute biomarker is an early biomarker that can be used to assess injury or suspected injury within the early stage of about 2 hours of injury or suspected injury.

[0092] "Identical" or "identity," as used herein in the context of two or more polypeptide or polynucleotide sequences, can mean that the sequences have a specified percentage of

residues that are the same over a specified region. The percentage can be calculated by optimally aligning the two sequences, comparing the two sequences over the specified region, determining the number of positions at which the identical residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the specified region, and multiplying the result by 100 to yield the percentage of sequence identity. In cases where the two sequences are of different lengths or the alignment produces one or more staggered ends and the specified region of comparison includes only a single sequence, the residues of the single sequence are included in the denominator but not the numerator of the calculation.

[0093] "Injury to the head" or "head injury" as used interchangeably herein, refers to any trauma to the scalp, skull, or brain. Such injuries may include only a minor bump on the skull or may be a serious brain injury. Such injuries include primary injuries to the brain and/or secondary injuries to the brain. Primary brain injuries occur during the initial insult and result from displacement of the physical structures of the brain. More specifically, a primary brain injury is the physical damage to parenchyma (tissue, vessels) that occurs during the traumatic event, resulting in shearing and compression of the surrounding brain tissue. Secondary brain injuries occur subsequent to the primary injury and may involve an array of cellular processes. More specifically, a secondary brain injury refers to the changes that evolve over a period of time (from hours to days) after the primary brain injury. It includes an entire cascade of cellular, chemical, tissue, or blood vessel changes in the brain that contribute to further destruction of brain tissue.

[0094] An injury to the head can be either closed or open (penetrating). A closed head injury refers to a trauma to the scalp, skull or brain where there is no penetration of the skull by a striking object. An open head injury refers a trauma to the scalp, skull or brain where there is penetration of the skull by a striking object. An injury to the head may be caused by physical shaking of a person, by blunt impact by an external mechanical or other force that results in a closed or open head trauma (e.g., vehicle accident such as with an automobile, plane, train, etc.; blow to the head such as with a baseball bat, or from a firearm), a cerebral vascular accident (e.g., stroke), one or more falls (e.g., as in sports or other activities), explosions or blasts (collectively, "blast injuries") and by other types of blunt force trauma. Alternatively, an injury to the head may be caused by the ingestion and/or exposure to a chemical, toxin or a combination of a chemical and toxin. Examples of such chemicals and/or toxins include fires, molds, asbestos, pesticides and insecticides, organic solvents, paints, glues, gases (such as carbon monoxide, hydrogen sulfide, and cyanide), organic

metals (such as methyl mercury, tetraethyl lead and organic tin) and/or one or more drugs of abuse. Alternatively, an injury to the head may be caused as a result of a subject suffering from an autoimmune disease, a metabolic disorder, a brain tumor, one or more viruses, meningitis, hydrocephalus, hypoxia or any combinations thereof. In some cases, it is not possible to be certain whether any such event or injury has occurred or taken place. For example, there may be no history on a patient or subject, the subject may be unable to speak, the subject may be aware of what events they were exposed to, etc. Such circumstances are described herein as the subject "may have sustained an injury to the head." In certain embodiments herein, the closed head injury does not include and specifically excludes a cerebral vascular accident, such as stroke.

[0095] "Isolated polynucleotide" as used herein may mean a polynucleotide (e.g., of genomic, cDNA, or synthetic origin, or a combination thereof) that, by virtue of its origin, the isolated polynucleotide is not associated with all or a portion of a polynucleotide with which the "isolated polynucleotide" is found in nature; is operably linked to a polynucleotide that it is not linked to in nature; or does not occur in nature as part of a larger sequence.

[0096] "Label" and "detectable label" as used herein refer to a moiety attached to an antibody or an analyte to render the reaction between the antibody and the analyte detectable, and the antibody or analyte so labeled is referred to as "detectably labeled." A label can produce a signal that is detectable by visual or instrumental means. Various labels include signal-producing substances, such as chromagens, fluorescent compounds, chemiluminescent compounds, radioactive compounds, and the like. Representative examples of labels include moieties that produce light, e.g., acridinium compounds, and moieties that produce fluorescence, *e.g.,* fluorescein. Other labels are described herein. In this regard, the moiety, itself, may not be detectable but may become detectable upon reaction with yet another moiety. Use of the term "detectably labeled" is intended to encompass such labeling.

[0097] Any suitable detectable label as is known in the art can be used. For example, the detectable label can be a radioactive label (such as 3H, 14C, 32P, 33P, 35S, 90Y, 99Tc, 111In, 125I, 131I, 177Lu, 166Ho, and 153Sm), an enzymatic label (such as horseradish peroxidase, alkaline peroxidase, glucose 6-phosphate dehydrogenase, and the like), a chemiluminescent label (such as acridinium esters, thioesters, or sulfonamides; luminol, isoluminol, phenanthridinium esters, and the like), a fluorescent label (such as fluorescein (e.g., 5-fluorescein, 6-carboxyfluorescein, 3'6-carboxyfluorescein, 5(6)-carboxyfluorescein, 6-hexachloro-fluorescein, 6-tetrachlorofluorescein, fluorescein isothiocyanate, and the like)), rhodamine, phycobiliproteins, R-phycoerythrin, quantum dots (e.g., zinc sulfide-capped

cadmium selenide), a thermometric label, or an immuno-polymerase chain reaction label. An introduction to labels, labeling procedures and detection of labels is found in Polak and Van Noorden, *Introduction to Immunocytochemistry*, 2nd ed., Springer Verlag, N.Y. (1997), and in Haugland, *Handbook of Fluorescent Probes and Research Chemicals* (1996), which is a combined handbook and catalogue published by Molecular Probes, Inc., Eugene, Oregon. A fluorescent label can be used in FPIA (see, e.g., U.S. Patent Nos. 5,593,896, 5,573,904, 5,496,925, 5,359,093, and 5,352,803, which are hereby incorporated by reference in their entireties). An acridinium compound can be used as a detectable label in a homogeneous chemiluminescent assay (see, e.g., Adamczyk *et al.*, Bioorg. *Med. Chem. Lett.* 16: 1324-1328 (2006); Adamczyk *et al.*, *Bioorg. Med. Chem. Lett.* 4: 2313-2317 (2004); Adamczyk *et al.*, *Biorg. Med. Chem. Lett.* 14: 3917-3921 (2004); and Adamczyk *et al.*, *Org. Lett.* 5: 3779-3782 (2003)).

[0098] In one aspect, the acridinium compound is an acridinium-9-carboxamide. Methods for preparing acridinium 9-carboxamides are described in Mattingly, *J. Biolumin. Chemilumin.* 6: 107-114 (1991); Adamczyk *et al.*, *J. Org. Chem.* 63: 5636-5639 (1998); Adamczyk *et al.*, *Tetrahedron* 55: 10899-10914 (1999); Adamczyk *et al.*, *Org. Lett.* 1: 779-781 (1999); Adamczyk *et al.*, *Bioconjugate Chem.* 11: 714-724 (2000); Mattingly *et al.*, *In Luminescence Biotechnology: Instruments and Applications*; Dyke, K. V. Ed.; CRC Press: Boca Raton, pp. 77–105 (2002); Adamczyk *et al.*, *Org. Lett.* 5: 3779-3782 (2003); and U.S. Patent Nos. 5,468,646, 5,543,524 and 5,783,699 (each of which is incorporated herein by reference in its entirety for its teachings regarding same).

[0099] Another example of an acridinium compound is an acridinium-9-carboxylate aryl ester. An example of an acridinium-9-carboxylate aryl ester of formula II is 10-methyl-9-(phenoxycarbonyl)acridinium fluorosulfonate (available from Cayman Chemical, Ann Arbor, MI). Methods for preparing acridinium 9-carboxylate aryl esters are described in McCapra *et al.*, Photochem. Photobiol. 4: 1111-21 (1965); Razavi *et al.*, *Luminescence* 15: 245-249 (2000); Razavi *et al.*, *Luminescence* 15: 239-244 (2000); and U.S. Patent No. 5,241,070 (each of which is incorporated herein by reference in its entirety for its teachings regarding same). Such acridinium-9-carboxylate aryl esters are efficient chemiluminescent indicators for hydrogen peroxide produced in the oxidation of an analyte by at least one oxidase in terms of the intensity of the signal and/or the rapidity of the signal. The course of the chemiluminescent emission for the acridinium-9-carboxylate aryl ester is completed rapidly, *i.e.*, in under 1 second, while the acridinium-9-carboxamide chemiluminescent emission extends over 2 seconds. Acridinium-9-carboxylate aryl ester, however, loses its

chemiluminescent properties in the presence of protein. Therefore, its use requires the absence of protein during signal generation and detection. Methods for separating or removing proteins in the sample are well-known to those skilled in the art and include, but are not limited to, ultrafiltration, extraction, precipitation, dialysis, chromatography, and/or digestion (see, *e.g.*, Wells, *High Throughput Bioanalytical Sample Preparation. Methods and Automation Strategies*, Elsevier (2003)). The amount of protein removed or separated from the test sample can be about 40%, about 45%, about 50%, about 55%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, or about 95%. Further details regarding acridinium-9-carboxylate aryl ester and its use are set forth in U.S. Patent App. No. 11/697,835, filed April 9, 2007. Acridinium-9-carboxylate aryl esters can be dissolved in any suitable solvent, such as degassed anhydrous N,N-dimethylformamide (DMF) or aqueous sodium cholate.

[0100] "Linking sequence" or "linking peptide sequence" refers to a natural or artificial polypeptide sequence that is connected to one or more polypeptide sequences of interest (*e.g.*, full-length, fragments, etc.). The term "connected" refers to the joining of the linking sequence to the polypeptide sequence of interest. Such polypeptide sequences are preferably joined by one or more peptide bonds. Linking sequences can have a length of from about 4 to about 50 amino acids. Preferably, the length of the linking sequence is from about 6 to about 30 amino acids. Natural linking sequences can be modified by amino acid substitutions, additions, or deletions to create artificial linking sequences. Linking sequences can be used for many purposes, including in recombinant Fabs. Exemplary linking sequences include, but are not limited to: (i) Histidine (His) tags, such as a 6X His tag, which has an amino acid sequence of HHHHHH (SEQ ID NO:3), are useful as linking sequences to facilitate the isolation and purification of polypeptides and antibodies of interest; (ii) Enterokinase cleavage sites, like His tags, are used in the isolation and purification of proteins and antibodies of interest. Often, enterokinase cleavage sites are used together with His tags in the isolation and purification of proteins and antibodies of interest. Various enterokinase cleavage sites are known in the art. Examples of enterokinase cleavage sites include, but are not limited to, the amino acid sequence of DDDDK (SEQ ID NO:4) and derivatives thereof (*e.g.*, ADDDDK (SEQ ID NO:5), etc.); (iii) Miscellaneous sequences can be used to link or connect the light and/or heavy chain variable regions of single chain variable region fragments. Examples of other linking sequences can be found in Bird *et al.*, *Science* 242: 423-426 (1988); Huston *et al.*, *PNAS USA* 85: 5879-5883 (1988); and McCafferty *et al.*, *Nature* 348: 552-554 (1990). Linking sequences also can be modified for

additional functions, such as attachment of drugs or attachment to solid supports. In the context of the present disclosure, the monoclonal antibody, for example, can contain a linking sequence, such as a His tag, an enterokinase cleavage site, or both.

[0101] "Monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigen. Furthermore, in contrast to polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological.

[0102] "MRI" as used herein refers to magnetic resonance imaging, which is a medical imaging technique used in radiology to form pictures of the anatomy and the physiological processes of the body in both health and disease. MRI scanners, which is based on the science of nuclear magnetic resonance (NMR), use strong magnetic fields, radio waves, and field gradients to generate images of the inside of the body.

[0103] "Multivalent binding protein" is used herein to refer to a binding protein comprising two or more antigen binding sites (also referred to herein as "antigen binding domains"). A multivalent binding protein is preferably engineered to have three or more antigen binding sites, and is generally not a naturally occurring antibody. The term "multispecific binding protein" refers to a binding protein that can bind two or more related or unrelated targets, including a binding protein capable of binding two or more different epitopes of the same target molecule.

[0104] "Negative predictive value" or "NPV" as used interchangeably herein refers to the probability that a subject has a negative outcome given that they have a negative test result.

[0105] "Point-of-care device" refers to a device used to provide medical diagnostic testing at or near the point-of-care (namely, outside of a laboratory), at the time and place of patient care (such as in a hospital, physician's office, urgent or other medical care facility, a patient's home, a nursing home and/or a long term care and/or hospice facility). Examples of point-of-

care devices include those produced by Abbott Laboratories (Abbott Park, IL) (*e.g.*, i-STAT and i-STAT Alinity, Universal Biosensors (Rowville, Australia) (see US 2006/0134713), Axis-Shield PoC AS (Oslo, Norway) and Clinical Lab Products (Los Angeles, USA).

[0106] "Positive predictive value" or "PPV" as used interchangeably herein refers to the probability that a subject has a positive outcome given that they have a positive test result.

[0107] "Quality control reagents" in the context of immunoassays and kits described herein, include, but are not limited to, calibrators, controls, and sensitivity panels. A "calibrator" or "standard" typically is used (e.g., one or more, such as a plurality) in order to establish calibration (standard) curves for interpolation of the concentration of an analyte, such as an antibody or an analyte. Alternatively, a single calibrator, which is near a reference level or control level (e.g., "low", "medium", or "high" levels), can be used. Multiple calibrators (*i.e.*, more than one calibrator or a varying amount of calibrator(s)) can be used in conjunction to comprise a "sensitivity panel."

[0108] A "receiver operating characteristic" curve or "ROC" curve refers to a graphical plot that illustrates the performance of a binary classifier system as its discrimination threshold is varied. For example, an ROC curve can be a plot of the true positive rate against the false positive rate for the different possible cutoff points of a diagnostic test. It is created by plotting the fraction of true positives out of the positives (TPR = true positive rate) vs. the fraction of false positives out of the negatives (FPR = false positive rate), at various threshold settings. TPR is also known as sensitivity, and FPR is one minus the specificity or true negative rate. The ROC curve demonstrates the tradeoff between sensitivity and specificity (any increase in sensitivity will be accompanied by a decrease in specificity); the closer the curve follows the left-hand border and then the top border of the ROC space, the more accurate the test; the closer the curve comes to the 45-degree diagonal of the ROC space, the less accurate the test; the slope of the tangent line at a cutoff point gives the likelihood ratio (LR) for that value of the test; and the area under the curve is a measure of text accuracy.

[0109] "Recombinant antibody" and "recombinant antibodies" refer to antibodies prepared by one or more steps, including cloning nucleic acid sequences encoding all or a part of one or more monoclonal antibodies into an appropriate expression vector by recombinant techniques and subsequently expressing the antibody in an appropriate host cell. The terms include, but are not limited to, recombinantly produced monoclonal antibodies, chimeric antibodies, humanized antibodies (fully or partially humanized), multi-specific or multi-valent structures formed from antibody fragments, bifunctional antibodies, heteroconjugate Abs, DVD-Ig®s, and other antibodies as described in (i) herein. (Dual-variable domain

immunoglobulins and methods for making them are described in Wu, C., *et al.*, *Nature Biotechnology*, 25:1290-1297 (2007)). The term "bifunctional antibody," as used herein, refers to an antibody that comprises a first arm having a specificity for one antigenic site and a second arm having a specificity for a different antigenic site, i.e., the bifunctional antibodies have a dual specificity.

[0110] "Reference level" as used herein refers to an assay cutoff value that is used to assess diagnostic, prognostic, or therapeutic efficacy and that has been linked or is associated herein with various clinical parameters (e.g., presence of disease, stage of disease, severity of disease, progression, non-progression, or improvement of disease, etc.). This disclosure provides exemplary reference levels. However, it is well-known that reference levels may vary depending on the nature of the immunoassay (e.g., antibodies employed, reaction conditions, sample purity, etc.) and that assays can be compared and standardized. It further is well within the ordinary skill of one in the art to adapt the disclosure herein for other immunoassays to obtain immunoassay-specific reference levels for those other immunoassays based on the description provided by this disclosure. Whereas the precise value of the reference level may vary between assays, the findings as described herein should be generally applicable and capable of being extrapolated to other assays.

[0111] In certain aspects described herein, the reference level is described as being determined by any assay having a certain specificity and sensitivity.

[0112] "Risk assessment," "risk classification," "risk identification," or "risk stratification" of subjects (*e.g.*, patients) as used herein refers to the evaluation of factors including biomarkers, to predict the risk of occurrence of future events including disease onset or disease progression, so that treatment decisions regarding the subject may be made on a more informed basis.

[0113] "Sample," "test sample," "specimen," "sample from a subject," and "patient sample" as used herein may be used interchangeable and may be a sample of blood such as whole blood, tissue, urine, serum, plasma, amniotic fluid, cerebrospinal fluid, placental cells or tissue, endothelial cells, leukocytes, or monocytes. The sample can be used directly as obtained from a patient or can be pre-treated, such as by filtration, distillation, extraction, concentration, centrifugation, inactivation of interfering components, addition of reagents, and the like, to modify the character of the sample in some manner as discussed herein or otherwise as is known in the art. In some embodiments, the sample is a whole blood sample. In some embodiments, the sample is a serum sample. In yet other embodiments, the sample is a plasma sample.

[0114] A variety of cell types, tissue, or bodily fluid may be utilized to obtain a sample. Such cell types, tissues, and fluid may include sections of tissues such as biopsy and autopsy samples, frozen sections taken for histologic purposes, blood (such as whole blood), plasma, serum, red blood cells, platelets, interstitial fluid, cerebral spinal fluid, etc. Cell types and tissues may also include lymph fluid, cerebrospinal fluid, a fluid collected by A tissue or cell type may be provided by removing a sample of cells from a human and a non-human animal, but can also be accomplished by using previously isolated cells (*e.g.*, isolated by another person, at another time, and/or for another purpose). Archival tissues, such as those having treatment or outcome history, may also be used. Protein or nucleotide isolation and/or purification may not be necessary.

[0115] "Sensitivity" refers to the proportion of subjects for whom the outcome is positive that are correctly identified as positive (e.g., correctly identifing those subjects with a disease or medical condition for which they are being tested). For example, this might include correctly identifying subjects as having a TBI from those who do not have a TBI, correctly identifying subjects having a moderate, severe, or moderate to severe TBI from those having a mild TBI, correctly identifying subjects as having a mild TBI from those having a moderate, severe, or moderate to severe TBI, correctly identifying subjects as having a moderate, severe, or moderate to severe TBI from those having no TBI or correctly identifying subjects as having a mild TBI from those having no TBI, etc.).

[0116] "Specificity" of an assay as used herein refers to the proportion of subjects for whom the outcome is negative that are correctly identified as negative (e.g., correctly identifying those subjects who do not have a disease or medical condition for which they are being tested). For example, this might include correctly identifying subjects having an TBI from those who do not have a TBI, correctly identifying subjects not having a moderate, severe, or moderate to severe TBI from those having a mild TBI, correctly identifying subjects as not having a mild TBI from those having a moderate, severe, or moderate to severe TBI or correctly identifying subjects as not having any TBI, or correctly identifying subjects as having a mild TBI from those having no TBI, etc.).

[0117] "Series of calibrating compositions" refers to a plurality of compositions comprising a known concentration of UCH-L1, wherein each of the compositions differs from the other compositions in the series by the concentration of UCH-L1.

[0118] "Solid phase" or "solid support" as used interchangeably herein, refers to any material that can be used to attach and/or attract and immobilize (1) one or more capture agents or capture specific binding partners, or (2) one or more detection agents or detection

specific binding partners. The solid phase can be chosen for its intrinsic ability to attract and immobilize a capture agent. Alternatively, the solid phase can have affixed thereto a linking agent that has the ability to attract and immobilize the (1) capture agent or capture specific binding partner, or (2) detection agent or detection specific binding partner. For example, the linking agent can include a charged substance that is oppositely charged with respect to the capture agent (*e.g.*, capture specific binding partner) or detection agent (*e.g.*, detection specific binding partner) itself or to a charged substance conjugated to the (1) capture agent or capture specific binding partner or (2) detection agent or detection specific binding partner. In general, the linking agent can be any binding partner (preferably specific) that is immobilized on (attached to) the solid phase and that has the ability to immobilize the (1) capture agent or capture specific binding partner, or (2) detection agent or detection specific binding partner through a binding reaction. The linking agent enables the indirect binding of the capture agent to a solid phase material before the performance of the assay or during the performance of the assay. For examples, the solid phase can be plastic, derivatized plastic, magnetic, or non-magnetic metal, glass or silicon, including, for example, a test tube, microtiter well, sheet, bead, microparticle, chip, and other configurations known to those of ordinary skill in the art.

[0119] "Specific binding" or "specifically binding" as used herein may refer to the interaction of an antibody, a protein, or a peptide with a second chemical species, wherein the interaction is dependent upon the presence of a particular structure (*e.g.*, an antigenic determinant or epitope) on the chemical species; for example, an antibody recognizes and binds to a specific protein structure rather than to proteins generally. If an antibody is specific for epitope "A", the presence of a molecule containing epitope A (or free, unlabeled A), in a reaction containing labeled "A" and the antibody, will reduce the amount of labeled A bound to the antibody.

[0120] "Specific binding partner" is a member of a specific binding pair. A specific binding pair comprises two different molecules, which specifically bind to each other through chemical or physical means. Therefore, in addition to antigen and antibody specific binding pairs of common immunoassays, other specific binding pairs can include biotin and avidin (or streptavidin), carbohydrates and lectins, complementary nucleotide sequences, effector and receptor molecules, cofactors and enzymes, enzymes and enzyme inhibitors, and the like. Furthermore, specific binding pairs can include members that are analogs of the original specific binding members, for example, an analyte-analog. Immunoreactive specific binding members include antigens, antigen fragments, and antibodies, including monoclonal and

polyclonal antibodies as well as complexes and fragments thereof, whether isolated or recombinantly produced.

[0121] "Statistically significant" as used herein refers to the likelihood that a relationship between two or more variables is caused by something other than random chance. Statistical hypothesis testing is used to determine whether the result of a data set is statistically significant. In statistical hypothesis testing, a statistical significant result is attained whenever the observed *p*-value of a test statistic is less than the significance level defined of the study. The *p*-value is the probability of obtaining results at least as extreme as those observed, given that the null hypothesis is true. Examples of statistical hypothesis analysis include Wilcoxon signed-rank test, t-test, Chi-Square or Fisher's exact test. "Significant" as used herein refers to a change that has not been determined to be statistically significant (e.g., it may not have been subject to statistical hypothesis testing).

[0122] "Subject" and "patient" as used herein interchangeably refers to any vertebrate, including, but not limited to, a mammal (*e.g.*, cow, pig, camel, llama, horse, goat, rabbit, sheep, hamsters, guinea pig, cat, dog, rat, and mouse, a non-human primate (for example, a monkey, such as a cynomolgous or rhesus monkey, chimpanzee, etc.) and a human). In some embodiments, the subject may be a human or a non-human. In some embodiments, the subject is a human. The subject or patient may be undergoing other forms of treatment. In some embodiments, the subject is a human that may be undergoing other forms of treatment.

[0123] "Treat," "treating" or "treatment" are each used interchangeably herein to describe reversing, alleviating, or inhibiting the progress of a disease and/or injury, or one or more symptoms of such disease, to which such term applies. Depending on the condition of the subject, the term also refers to preventing a disease, and includes preventing the onset of a disease, or preventing the symptoms associated with a disease. A treatment may be either performed in an acute or chronic way. The term also refers to reducing the severity of a disease or symptoms associated with such disease prior to affliction with the disease. Such prevention or reduction of the severity of a disease prior to affliction refers to administration of a pharmaceutical composition to a subject that is not at the time of administration afflicted with the disease. "Preventing" also refers to preventing the recurrence of a disease or of one or more symptoms associated with such disease. "Treatment" and "therapeutically," refer to the act of treating, as "treating" is defined above.

[0124] "Traumatic Brain Injury" or "TBI" as used interchangeably herein refers to a complex injury with a broad spectrum of symptoms and disabilities. TBI is most often an acute event similar to other injuries. TBI can be classified as "mild," "moderate," "severe",

or "moderate to severe". The causes of TBI are diverse and include, for example, physical shaking by a person, a car accident, injuries from firearms, cerebral vascular accidents (e.g., strokes), falls, explosions or blasts and other types of blunt force trauma. Other causes of TBI include the ingestion and/or exposure to one or more chemicals or toxins (such as fires, molds, asbestos, pesticides and insecticides, organic solvents, paints, glues, gases (such as carbon monoxide, hydrogen sulfide, and cyanide), organic metals (such as methyl mercury, tetraethyl lead and organic tin), one or more drugs of abuse or combinations thereof). Alternatively, TBI can occur in subjects suffering from an autoimmune disease, a metabolic disorder, a brain tumor, hypoxia, one or more viruses, meningitis, hydrocephalus or combinations thereof. Young adults and the elderly are the age groups at highest risk for TBI. In certain embodiments herein, traumatic brain injury or TBI does not include and specifically excludes cerebral vascular accidents such as strokes.

[0125] "Mild TBI" as used herein refers to a brain injury where loss of consciousness is brief and usually a few seconds or minutes and/or confusion and disorientation is shorter than 1 hour. Mild TBI is also referred to as a concussion, minor head trauma, minor TBI, minor brain injury, and minor head injury. While MRI and CT scans are often normal, the individual with mild TBI may have cognitive problems such as headache, difficulty thinking, memory problems, attention deficits, mood swings and frustration.

[0126] Mild TBI is the most prevalent TBI and is often missed at time of initial injury. Typically, a subject has a Glasgow Coma scale number of between 13-15 (such as 13-15 or 14-15). Fifteen percent (15%) of people with mild TBI have symptoms that last 3 months or more. Mild TBI is defined as the result of the forceful motion of the head or impact causing a brief change in mental status (confusion, disorientation or loss of memory) or loss of consciousness for less than 30 minutes. Common symptoms of mild TBI include fatigue, headaches, visual disturbances, memory loss, poor attention/concentration, sleep disturbances, dizziness/loss of balance, irritability-emotional disturbances, feelings of depression, and seizures. Other symptoms associated with mild TBI include nausea, loss of smell, sensitivity to light and sounds, mood changes, getting lost or confused, and/or slowness in thinking.

[0127] "Moderate TBI" as used herein refers to a brain injury where loss of consciousness and/or confusion and disorientation is between 1 and 24 hours and the subject has a Glasgow Coma scale number of between 9-13 (such as 9-12 or 9-13). The individual with moderate TBI have abnormal brain imaging results. "Severe TBI" as used herein refers to a brain injury where loss of consciousness is more than 24 hours and memory loss after the injury or

penetrating skull injury longer than 24 hours and the subject has a Glasgow Coma scale number between 3-8. The deficits range from impairment of higher level cognitive functions to comatose states. Survivors may have limited function of arms or legs, abnormal speech or language, loss of thinking ability or emotional problems. Individuals with severe injuries can be left in long-term unresponsive states. For many people with severe TBI, long-term rehabilitation is often necessary to maximize function and independence.

[0128] "Moderate to severe" TBI as used herein refers to a spectrum of brain injury that includes moderate to severe TBI and thus encompasses moderate TBI alone, severe TBI and moderate to severe TBI combined. For example, in some clinical situations, a subject may initially be diagnosed as having a moderate TBI but who, over the course of time (minutes, hours or days), progress to having a severe TBI (such, as for example, in situations when there is a brain bleed). Such subjects would be examples of patients that could be classified as "moderate to severe". Common symptoms of moderate to severe TBI include cognitive deficits including difficulties with attention, concentration, distractibility, memory, speed of processing, confusion, perseveration, impulsiveness, language processing, and/or "executive functions", not understanding the spoken word (receptive aphasia), difficulty speaking and being understood (expressive aphasia), slurred speech, speaking very fast or very slow, problems reading, problems writing, difficulties with interpretation of touch, temperature, movement, limb position and fine discrimination, the integration or patterning of sensory impressions into psychologically meaningful data, partial or total loss of vision, weakness of eye muscles and double vision (diplopia), blurred vision, problems judging distance, involuntary eye movements (nystagmus), intolerance of light (photophobia), hearing, such as decrease or loss of hearing, ringing in the ears (tinnitus), increased sensitivity to sounds, loss or diminished sense of smell (anosmia), loss or diminished sense of taste, the convulsions associated with epilepsy that can be several types and can involve disruption in consciousness, sensory perception, or motor movements, control of bowel and bladder, sleep disorders, loss of stamina, appetite changes, regulation of body temperature, menstrual difficulties, dependent behaviors, emotional ability, lack of motivation, irritability, aggression, depression, disinhibition, or denial/lack of awareness. Subjects having a moderate to severe TBI can have a Glasgow Coma scale score from 3-12 (which includes the range of 9-13 for a moderate TBI, and 3-8 for a severe TBI).

[0129] "Ubiquitin carboxy-terminal hydrolase L1" or "UCH-L1" as used interchangeably herein refers to a deubiquitinating enzyme encoded by the *UCH-L1* gene in humans. UCH-L1, also known as ubiquitin carboxyl-terminal esterase L1 and ubiquitin thiolesterase, is a

member of a gene family whose products hydrolyze small C-terminal adducts of ubiquitin to generate the ubiquitin monomer.

[0130] "UCH-L1 status" can mean either the level or amount of UCH-L1 at a point in time (such as with a single measure of UCH-L1), the level or amount of UCH-L1 associated with monitoring (such as with a repeat test on a subject to identify an increase or decrease in UCH-L1 amount), the level or amount of UCH-L1 associated with treatment for traumatic brain injury (whether a primary brain injury and/or a secondary brain injury) or combinations thereof.

[0131] "Variant" is used herein to describe a peptide or polypeptide that differs in amino acid sequence by the insertion, deletion, or conservative substitution of amino acids, but retain at least one biological activity. Representative examples of "biological activity" include the ability to be bound by a specific antibody or to promote an immune response. Variant is also used herein to describe a protein with an amino acid sequence that is substantially identical to a referenced protein with an amino acid sequence that retains at least one biological activity. A conservative substitution of an amino acid, *i.e.*, replacing an amino acid with a different amino acid of similar properties (*e.g.*, hydrophilicity, degree, and distribution of charged regions) is recognized in the art as typically involving a minor change. These minor changes can be identified, in part, by considering the hydropathic index of amino acids, as understood in the art. Kyte *et al.*, *J. Mol. Biol.* 157:105-132 (1982). The hydropathic index of an amino acid is based on a consideration of its hydrophobicity and charge. It is known in the art that amino acids of similar hydropathic indexes can be substituted and still retain protein function. In one aspect, amino acids having hydropathic indexes of ±2 are substituted. The hydrophilicity of amino acids can also be used to reveal substitutions that would result in proteins retaining biological function. A consideration of the hydrophilicity of amino acids in the context of a peptide permits calculation of the greatest local average hydrophilicity of that peptide, a useful measure that has been reported to correlate well with antigenicity and immunogenicity. U.S. Patent No. 4,554,101, incorporated fully herein by reference. Substitution of amino acids having similar hydrophilicity values can result in peptides retaining biological activity, for example immunogenicity, as is understood in the art. Substitutions may be performed with amino acids having hydrophilicity values within ±2 of each other. Both the hydrophobicity index and the hydrophilicity value of amino acids are influenced by the particular side chain of that amino acid. Consistent with that observation, amino acid substitutions that are compatible with biological function are understood to depend on the relative similarity of the amino

acids, and particularly the side chains of those amino acids, as revealed by the hydrophobicity, hydrophilicity, charge, size, and other properties. "Variant" also can be used to refer to an antigenically reactive fragment of an anti-UCH-L1 antibody that differs from the corresponding fragment of anti-UCH-L1 antibody in amino acid sequence but is still antigenically reactive and can compete with the corresponding fragment of anti-UCH-L1 antibody for binding with UCH-L1. "Variant" also can be used to describe a polypeptide or a fragment thereof that has been differentially processed, such as by proteolysis, phosphorylation, or other post-translational modification, yet retains its antigen reactivity.

[0132] "Vector" is used herein to describe a nucleic acid molecule that can transport another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double-stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors can replicate autonomously in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors" (or simply, "expression vectors"). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. "Plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector. However, other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions, can be used. In this regard, RNA versions of vectors (including RNA viral vectors) may also find use in the context of the present disclosure.

[0133] Unless otherwise defined herein, scientific and technical terms used in connection with the present disclosure shall have the meanings that are commonly understood by those of ordinary skill in the art. For example, any nomenclatures used in connection with, and techniques of, cell and tissue culture, molecular biology, immunology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those that are well known and commonly used in the art. The meaning and scope of the terms should be clear; in the event, however of any latent ambiguity, definitions provided herein take precedent over any dictionary or extrinsic definition. Further, unless otherwise

required by context, singular terms shall include pluralities and plural terms shall include the singular.

2. Methods of Aiding in the Diagnosis and Evaluation of Whether a Human Subject has Sustained an Injury to the Head Using a Reference Level

[0134] The present disclosure relates, among other methods, to a method of evaluating or aiding in the diagnosis and evaluation of whether a human subject has sustained or may have sustained an injury to the head. The method can aid in determining the extent of traumatic brain injury in a human subject with a suspected injury to the head, e.g., determining whether the subject has mild traumatic brain injury or moderate to severe traumatic brain injury, or a mild traumatic brain injury, moderate traumatic brain injury, severe traumatic brain injury, or a moderate to severe traumatic brain injury. As used here, "determining whether the subject has a mild traumatic brain injury or moderate to severe traumatic brain injury" or "determining whether the subject has a mild traumatic brain injury, a moderate traumatic brain injury, a severe traumatic brain injury, or a moderate to severe brain injury" refers to the fact that the aforementioned method can be used, e.g., with other information (e.g., clinical assessment data), to determine that the subject is more likely than not to have mild traumatic brain injury or moderate to severe traumatic brain injury, or a mild traumatic brain injury, moderate traumatic brain injury, severe traumatic brain injury, or moderate to severe traumatic brain injury. The method can include performing an assay on a sample obtained from the human subject within about 2 hours after a suspected injury to the head to measure or detect a level of an early biomarker of traumatic brain injury, such as ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), glial fibrillary acidic protein (GFAP), or a combination thereof, in the sample and determining whether the subject has sustained a mild or a moderate to severe traumatic brain injury (TBI). In some embodiments, the subject is determined as having (1) a moderate or severe TBI when the level of the early biomarker in the sample is higher than a reference level of the early biomarker, or (2) a mild TBI when the level of the early biomarker in the sample is lower than a reference level of the early biomarker. In yet other embodiments, the subject is determined as having (1) a moderate, severe, or moderate to severe TBI when the level of the early biomarker in the sample is higher than a reference level of the early biomarker, or (2) a mild TBI when the level of the early biomarker in the sample is lower than a reference level of the early biomarker. The sample can be a biological sample.

[0135] In some embodiments, the method can include obtaining a sample within about 2 hours of a suspected injury to the subject and contacting the sample with an antibody for an early biomarker of TBI, such as ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), glial fibrillary acidic protein (GFAP), or a combination thereof, to allow formation of a complex of the antibody and the early biomarker. The method also includes detecting the resulting antibody-early biomarker complex.

[0136] In some embodiments, the sample is taken from the human subject within about 2 hours of injury or suspected injury to the head. For example, the sample can be taken from the human subject within about 0 minutes, about 1 minute, about 2 minutes, about 3 minutes, about 4 minutes, about 5 minutes, about 6 minutes, about 7 minutes, about 8 minutes, about 9 minutes, about 10 minutes, about 11 minutes, about 12 minutes, about 13 minutes, about 14 minutes, about 15 minutes, about 20 minutes, about 30 minutes, about 60 minutes, about 90 minutes, or about 2 hours of injury or suspected injury to the head. In some embodiments, the onset of the presence of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, appears within about 0 minutes, about 1 minute, about 2 minutes, about 3 minutes, about 4 minutes, about 5 minutes, about 6 minutes, about 7 minutes, about 8 minutes, about 9 minutes, about 10 minutes, about 11 minutes, about 12 minutes, about 13 minutes, about 14 minutes, about 15 minutes, about 20 minutes, about 30 minutes, about 60 minutes, about 90 minutes, or about 2 hours after injury to the head.

[0137] In some embodiments, the subject has received a Glasgow Coma Scale score before or after the assay is performed. In some embodiments, the subject is suspected as having moderate or severe traumatic brain injury or a moderate to severe traumatic brain injury based on the Glasgow Coma Scale score. In some embodiments, the reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, is correlated with subjects having moderate to severe traumatic brain injury. In some embodiments, the reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, is correlated with a Glasgow Coma Scale score of 9-13 (a moderate TBI). In some embodiments, the reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, is correlated with a Glasgow Coma Scale score of 3-8 (a severe TBI). In some embodiments, the reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, is correlated with a Glasgow Coma Scale score of 3-12 (a moderate to severe TBI). In some embodiments, the subject is suspected as having mild traumatic brain injury based on the Glasgow Coma Scale score. In some embodiments, the reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, is correlated with

subjects having mild traumatic brain injury. In some embodiments, the reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, is correlated with a Glasgow Coma Scale score of 13-15 (mild TBI).

[0138] Generally, a reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, can also be employed as a benchmark against which to assess results obtained upon assaying a test sample for the early biomarker, such as UCH-L1, GFAP, or a combination thereof. Generally, in making such a comparison, the reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, is obtained by running or conducting a particular assay a sufficient number of times and under appropriate conditions such that a linkage or association of analyte presence, amount or concentration with a particular stage or endpoint of TBI or with particular indicia can be made. Typically, the reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, is obtained with assays of reference subjects (or populations of subjects). The early biomarker, such as UCH-L1, GFAP, or a combination thereof, measured can include fragments thereof, degradation products thereof, and/or enzymatic cleavage products thereof.

[0139] In certain embodiments, the reference level may be correlated with control subjects that have not sustained a head injury.

[0140] In some embodiments, the reference level for UCH-L1 is about 73.5 pg/mL. In some embodiments, the reference level for UCH-L1 is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 4.0 fold, 4.5 fold, or 5.0 fold greater than about 73.5 pg/mL.

[0141] In some embodiments, the reference level for UCH-L1 is about 88.2 pg/mL. In some embodiments, the reference level for UCH-L1 is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 4.0 fold, 4.5 fold, or 5.0 fold greater than 88.2 pg/mL.

[0142] In some embodiments, the reference level for UCH-L1 is about 371 pg/mL. In some embodiments, the reference level for UCH-L1 is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 4.0 fold, 4.5 fold, or 5.0 fold greater than about 371 pg/mL.

[0143] In some embodiments, the reference level for GFAP is about 9.0 pg/mL. In some embodiments, the reference level for GFAP is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 4.0 fold, 4.5 fold, or 5.0 fold greater than about 9.0 pg/mL.

[0144] In some embodiments, the reference level for GFAP is about11.7 pg/mL. In some embodiments, the reference level for GFAP is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 4.0 fold, 4.5 fold, or 5.0 fold greater than about 11.7 pg/mL.

[0145] In some embodiments, the reference level for GFAP is about 371 pg/mL. In some embodiments, the reference level for GFAP is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 4.0 fold,

4.5 fold, or 5.0 fold greater than about 371 pg/mL. In some embodiments, the reference level can be any one of the values or range of values shown in Tables 2 and/or 3.

Table 2 UCH-L1 Levels

Control (pg/mL)	Fold change Reference Compared to Control							
	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
73.5	110.25	147.0	183.75	220.5	257.25	294.0	330.75	367.5
88.2	132.3	176.4	220.5	264.6	308.7	352.8	396.9	441.0
371	556.50	742.0	927.5	1113	1298.5	1484.0	1669.5	1855.0

Table 3 GFAP Levels

Control (pg/mL)	Fold change Reference Compared to Control							
	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
9.0	13.50	18.00	22.50	27.00	31.50	36.00	40.50	45.00
11.7	17.55	23.40	29.25	35.10	40.95	46.80	52.65	58.50
42	63.00	84.00	105.0	126.0	147.0	168.0	189.0	210.0

[0146] The data in Tables 2 and 3 shown was derived from the results described in Example 3.

[0147] In some embodiments, the sample is taken (a) within about 10 minutes after the suspected injury and the reference level of UCH-L1 is at least about 1669 pg/mL; (b) within about 12 minutes after the suspected injury and the reference level of UCH-L1 is at least about 3285 pg/mL; (c) within about 20 minutes after the suspected injury and the reference level of UCH-L1 is at least about 2919 pg/mL; (d) within about 10 minutes after the suspected injury and the reference level of GFAP is at least about 108 pg/mL; (e) within about 12 minutes after the suspected injury and the reference level of GFAP is at least about 54 pg/mL; (f) within about 20 minutes after the suspected injury and the reference level of GFAP is at least about 1809 pg/mL; (g) within about 10 minutes after the suspected injury and the reference level of UCH-L1 is at least about 1669 pg/mL and the reference level of GFAP is at least about 108 pg/mL; (h) within about 12 minutes after the suspected injury and the reference level of UCH-L1 is at least about 3285 pg/mL and the reference level of GFAP is at least about 54 pg/mL; or (i) within about 20 minutes after the suspected injury and the reference level of UCH-L1 is at least about 2919 pg/mL and the reference level of GFAP is at least about 1809 pg/mL.

[0148] In some embodiments, the reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, is determined by an assay having a sensitivity of between at least about 65% to about 100% and a specificity of between at least about 30% to about 100%. In some embodiments, the sensitivity is between at least about 65% to about 100%,

between at least about 65% to at least about 99%, between at least about 65% to at least about 95%, between at least about 65% to at least about 90%, between at least about 65% to at least about 85%, between at least about 65% to at least about 80%, between at least about 65% to at least about 75%, between at least about 65% to at least about 70%, between at least about 75% to about 100%, between at least about 75% to at least about 99%, between at least about 75% to at least about 95%, between at least about 75% to at least about 90%, between at least about 75% to at least about 85%, between at least about 75% to at least about 80%, between at least about 85% to about 100%, between at least about 85% to at least about 99%, between at least about 85% to at least about 95%, between at least about 85% to at least about 90%, between at least about 95% to about 100%, or between at least about 95% to at least about 99%. In some embodiments, the sensitivity is at least about 65.0%, at least about 70.0%, at least about 75.0%, at least about 80.0%, at least about 85.0%, at least about 87.5%, at least about 90.0%, at least about 95.0%, at least about 99.0%, at least about 99.1%, at least about 99.2%, at least about 99.3%, at least about 99.4%, at least about 99.5%, at least about 99.6%, at least about 99.7%, at least about 99.8%, at least about 99.9%, or at least about 100.0%.

[0149] In some embodiments, the specificity is between at least about 30% to about 100%, between at least about 30% to about 99%, between at least about 30% to about 95%, between at least about 30% to about 90%, between at least about 30% to about 85%, between at least about 30% to about 80%, between at least about 30% to about 75%, between at least about 30% to about 70%, between at least about 30% to about 60%, between at least about 30% to about 50%, between at least about 40% to about 100%, between at least about 40% to about 99%, between at least about 40% to about 95%, between at least about 40% to about 90%, between at least about 40% to about 85%, between at least about 40% to about 80%, between at least about 40% to about 75%, between at least about 40% to about 70%, between at least about 40% to about 60%, between at least about 40% to about 50%, between at least about 50% to about 100%, between at least about 50% to about 99%, between at least about 50% to about 95%, between at least about 50% to about 90%, between at least about 50% to about 85%, between at least about 50% to about 80%, between at least about 50% to about 75%, between at least about 50% to about 70%, between at least about 50% to about 60%, between at least about 60% to about 100%, between at least about 60% to about 99%, between at least about 60% to about 95%, between at least about 60% to about 90%, between at least about 60% to about 85%, between at least about 60% to about 80%, between at least about 60% to about 75%, between at least about 60% to about 70%, between at least about 70% to about 100%, between at least about 70% to about 99%, between at least about 70% to about 95%,

between at least about 70% to about 90%, between at least about 70% to about 85%, between at least about 70% to about 80%, between at least about 70% to about 75%, between at least about 80% to about 100%, between at least about 80% to about 99%, between at least about 80% to about 95%, between at least about 80% to about 90%, between at least about 80% to about 85%, between at least about 90% to about 100%, between at least about 90% to about 99%, between at least about 90% to about 95%, between at least about 95% to about 99%, or between at least about 95% to about 100. In some embodiments, the specificity is at least about 30.0%, at least about 31.0%, at least about 32.0%, at least about 33.0%, at least about 34.0%, at least about 35.0%, at least about 36.0%, at least about 37.0%, at least about 38.0%, at least about 39.0%, at least about 40.0%, at least about 45.0%, at least about 50.0%, at least about 55.0%, at least about 60.0%, at least about 65.0%, at least about 70.0%, at least about 75.0%, at least about 80.0%, at least about 85.0%, at least about 90.0%, at least about 91.0%, at least about 92.0%, at least about 93.0%, at least about 94.0%, at least about 95.0%, at least about 96.0%, at least about 97.0%, at least about 98.0%, at least about 99.0%, at least about 99.1%, at least about 99.2%, at least about 99.3%, at least about 99.4%, at least about 99.5%, at least about 99.6%, at least about 99.7%, at least about 99.8%, at least about 99.9%, or at least about 100.0%. For example, the sensitivity is at least about 99% and the specificity is at least about 75%, the sensitivity is at least about 99% and the specificity is at least about 99%, or the sensitivity is at least about 100% and the specificity is at least about 100%.

[0150] In some embodiments, the reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, can be between at least about 5 pg/mL to about 3500 pg/mL. In some embodiments, the reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, can be between at least about 5 pg/mL to about 3500 pg/mL, between at least about 5 pg/mL to about 3000 pg/mL, between at least about 5 pg/mL to about 2500 pg/mL, between at least about 5 pg/mL to about 2000 pg/mL, between at least about 5 pg/mL to about 1500 pg/mL, between at least about 5 pg/mL to about 1000 pg/mL, between at least about 5 pg/mL to about 900 pg/mL, between at least about 5 pg/mL to about 800 pg/mL, between at least about 5 pg/mL to about 700 pg/mL, between at least about 5 pg/mL to about 600 pg/mL, between at least about 5 pg/mL to about 500 pg/mL, between at least about 10 pg/mL to about 3500 pg/mL, between at least about 10 pg/mL to about 3000 pg/mL, between at least about 10 pg/mL to about 2500 pg/mL, between at least about 10 pg/mL to about 2000 pg/mL, between at least about 10 pg/mL to about 1500 pg/mL, between at least about 10 pg/mL to about 1000 pg/mL, between at least about 10 pg/mL to about 900 pg/mL, between at least about 10 pg/mL to about 800 pg/mL, between at least about 10

pg/mL to about 700 pg/mL, between at least about 10 pg/mL to about 600 pg/mL, between at least about 10 pg/mL to about 500 pg/mL, between at least about 50 pg/mL to about 3500 pg/mL, between at least about 50 pg/mL to about 3000 pg/mL, between at least about 50 pg/mL to about 2500 pg/mL, between at least about 50 pg/mL to about 2000 pg/mL, between at least about 50 pg/mL to about 1500 pg/mL, between at least about 50 pg/mL to about 1000 pg/mL, between at least about 50 pg/mL to about 900 pg/mL, between at least about 50 pg/mL to about 800 pg/mL, between at least about 50 pg/mL to about 700 pg/mL, between at least about 50 pg/mL to about 600 pg/mL, between at least about 50 pg/mL to about 500 pg/mL, between at least about 100 pg/mL to about 3500 pg/mL, between at least about 100 pg/mL to about 3000 pg/mL, between at least about 100 pg/mL to about 2500 pg/mL, between at least about 100 pg/mL to about 2000 pg/mL, between at least about 100 pg/mL to about 1500 pg/mL, between at least about 100 pg/mL to about 1000 pg/mL, between at least about 100 pg/mL to about 900 pg/mL, between at least about 100 pg/mL to about 800 pg/mL, between at least about 100 pg/mL to about 700 pg/mL, between at least about 100 pg/mL to about 600 pg/mL, between at least about 100 pg/mL to about 500 pg/mL, between at least about 150 pg/mL to about 3500 pg/mL, between at least about 150 pg/mL to about 3000 pg/mL, between at least about 150 pg/mL to about 2500 pg/mL, between at least about 150 pg/mL to about 2000 pg/mL, between at least about 150 pg/mL to about 1500 pg/mL, between at least about 150 pg/mL to about 1000 pg/mL, between at least about 150 pg/mL to about 900 pg/mL, between at least about 150 pg/mL to about 800 pg/mL, between at least about 150 pg/mL to about 700 pg/mL, between at least about 150 pg/mL to about 600 pg/mL, between at least about 150 pg/mL to about 500 pg/mL, between at least about 200 pg/mL to about 3500 pg/mL, between at least about 200 pg/mL to about 3000 pg/mL, between at least about 200 pg/mL to about 2500 pg/mL, between at least about 200 pg/mL to about 2000 pg/mL, between at least about 200 pg/mL to about 1500 pg/mL, between at least about 200 pg/mL to about 1000 pg/mL, between at least about 200 pg/mL to about 900 pg/mL, between at least about 200 pg/mL to about 800 pg/mL, between at least about 200 pg/mL to about 700 pg/mL, between at least about 200 pg/mL to about 600 pg/mL, between at least about 200 pg/mL to about 500 pg/mL, between at least about 300 pg/mL to about 3500 pg/mL, between at least about 300 pg/mL to about 3000 pg/mL, between at least about 300 pg/mL to about 2500 pg/mL, between at least about 300 pg/mL to about 2000 pg/mL, between at least about 300 pg/mL to about 1500 pg/mL, between at least about 300 pg/mL to about 1000 pg/mL, between at least about 300 pg/mL to about 900 pg/mL, between at least about 300 pg/mL to about 800 pg/mL, between at least about 300 pg/mL to about 700 pg/mL, between at least

about 300 pg/mL to about 600 pg/mL, between at least about 300 pg/mL to about 500 pg/mL, between at least about 400 pg/mL to about 3500 pg/mL, between at least about 400 pg/mL to about 3000 pg/mL, between at least about 400 pg/mL to about 2500 pg/mL, between at least about 400 pg/mL to about 2000 pg/mL, between at least about 400 pg/mL to about 1500 pg/mL, between at least about 400 pg/mL to about 1000 pg/mL, between at least about 400 pg/mL to about 900 pg/mL, between at least about 400 pg/mL to about 800 pg/mL, between at least about 400 pg/mL to about 700 pg/mL, between at least about 400 pg/mL to about 600 pg/mL, between at least about 400 pg/mL to about 500 pg/mL, between at least about 500 pg/mL to about 3500 pg/mL, between at least about 500 pg/mL to about 3000 pg/mL, between at least about 500 pg/mL to about 2500 pg/mL, between at least about 500 pg/mL to about 2000 pg/mL, between at least about 500 pg/mL to about 1500 pg/mL, between at least about 500 pg/mL to about 1000 pg/mL, between at least about 500 pg/mL to about 900 pg/mL, between at least about 500 pg/mL to about 800 pg/mL, between at least about 500 pg/mL to about 700 pg/mL, between at least about 500 pg/mL to about 600 pg/mL, between at least about 1000 pg/mL to about 3500 pg/mL, between at least about 1000 pg/mL to about 3000 pg/mL, between at least about 1000 pg/mL to about 2500 pg/mL, between at least about 1000 pg/mL to about 2000 pg/mL, between at least about 1000 pg/mL to about 1500 pg/mL, between at least about 2000 pg/mL to about 3500 pg/mL, between at least about 2000 pg/mL to about 3000 pg/mL, or between at least about 2000 pg/mL to about 2500 pg/mL. For example, the reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, can be between at least about 10 pg/mL to about 1000 pg/mL, between at least about 70 pg/mL to about 3500 pg/mL, or between at least about 5 pg/mL to about 2000 pg/mL. For example, the reference level for UCH-L1 can be between at least about 70 pg/mL to about 3500 pg/mL and the reference level for GFAP can be between at least about 5 pg/mL to about 2000 pg/mL.

[0151] In some embodiments, the reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, can be at least about 5 pg/mL, can be at least about 10 pg/mL, at least about 20 pg/mL, at least about 30 pg/mL, at least about 40 pg/mL, at least about 50 pg/mL, at least about 55 pg/mL, at least about 60 pg/mL, at least about 65 pg/mL, at least about 70 pg/mL, at least about 75 pg/mL, at least about 80 pg/mL, at least about 85 pg/mL, at least about 90 pg/mL, at least about 95 pg/mL, at least about 100 pg/mL, at least about 150 pg/mL, at least about 200 pg/mL, at least about 250 pg/mL, at least about 300 pg/mL, at least about 350 pg/mL, at least about 400 pg/mL, at least about 450 pg/mL, at least about 500 pg/mL, at least about 550 pg/mL, at least about 600 pg/mL, at least abo700 pg/mL,

at least about 800 pg/mL, at least about 900 pg/mL, at least about 1000 pg/mL, at least about 1500 pg/mL, at least about 2000 pg/mL, at least about 2500 pg/mL, at least about 3000 pg/mL, or at least about 3500 pg/mL.

[0152] In some embodiments, the method further includes treating a human subject assessed as having moderate to severe traumatic brain injury with a traumatic brain injury treatment, as described below. In yet other embodiments, the method further includes treating a human subject assessed with moderate traumatic brain injury with traumatic brain injury treatment, as described below. In yet other embodiments, the method further includes treating a subject assessed with severe traumatic brain injury with a traumatic brain injury treatment. In some embodiments, the method further includes monitoring a human subject assessed as having mild traumatic brain injury, as described below. In other embodiments, the method further includes monitoring a human subject assessed as having a moderate traumatic brain injury, as described below. In yet other embodiments, the method further includes monitoring a human subject assessed as having a severe traumatic brain injury, as described below. In yet other embodiments, the method further includes monitoring a human subject assessed as having a moderate to severe traumatic brain injury.

[0153] The nature of the assay employed in the methods described herein is not critical and the test can be any assay known in the art such as, for example, immunoassays, protein immunoprecipitation, immunoelectrophoresis, chemical analysis, SDS-PAGE and Western blot analysis, or protein immunostaining, electrophoresis analysis, a protein assay, a competitive binding assay, a functional protein assay, or chromatography or spectrometry methods, such as high-performance liquid chromatography (HPLC) or liquid chromatography–mass spectrometry (LC/MS). Nonetheless, tests or assays competent to perform the claimed methods will be employed, such as, for example, assays having various sensitivities and sensitivities as described herein. Moreover, the assays employed in the methods described herein can be employed in a clinical chemistry format such as would be known by one of ordinary skill in the art. Such assays are described in further detail herein in Sections 5-9. It is known in the art that the values (e.g., reference levels, cutoffs, thresholds, specificities, sensitivities, concentrations of calibrators and/or controls etc.) used in an assay that employs specific sample type (e.g., such as an immunoassay that utilizes serum or a point-of-care device that employs whole blood) can be extrapolated to other assay formats using known techniques in the art, such as assay standardization. For example, one way in which assay standardization can be performed is by applying a factor to the calibrator employed in the assay to make the sample concentration read higher or lower to get a slope

that aligns with the comparator method. Other methods of standardizing results obtained on one assay to another assay are well known and have been described in the literature (See, for example, David Wild, *Immunoassay Handbook*, 4th edition, chapter 3.5, pages 315-322, the contents of which are herein incorporated by reference).

3. Methods of Aiding in the Determination of Whether to Perform a CT scan on a Human Subject Who Has Sustained an Injury to the Head Using a Reference Level

[0154] The present disclosure relates, among other methods, to a method of aiding in determining whether to perform a computerized tomography (CT) scan on a human subject who has sustained or may have sustained a suspected injury to the head. As used here, "determination of whether to perform a CT scan on a human subject" refers to the fact that the aforementioned method can be used, e.g., with other information (e.g., clinical assessment data), to determine that the subject is more likely than not to have a positive head CT scan. Specifically, such a method can comprise the steps of: (a) performing an assay on a sample obtained from the subject within about 2 hours after a suspected injury to the head to measure or detect a level of an early biomarker in the sample, said early biomarker comprising ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), glial fibrillary acidic protein (GFAP), or a combination thereof, in the sample; and (b) performing a CT scan on the subject when the level of the early biomarker in the sample is higher than a reference level of the early biomarker and not performing a CT scan on the subject when the level of the early biomarker in the sample is lower than a reference level of the early biomarker. The sample can be a biological sample.

[0155] In some embodiments, the method can include obtaining a sample within about 2 hours of a suspected injury to the subject and contacting the sample with an antibody for an early biomarker of TBI, such as ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), glial fibrillary acidic protein (GFAP), or a combination thereof, to allow formation of a complex of the antibody and the early biomarker. The method also includes detecting the resulting antibody-early biomarker complex.

[0156] In some embodiments, the sample is taken from the human subject within about 2 hours of injury or suspected injury to the head. For example, the sample can be taken from the human subject within about 0 minutes, about 1 minute, about 2 minutes, about 3 minutes, about 4 minutes, about 5 minutes, about 6 minutes, about 7 minutes, about 8 minutes, about 9 minutes, about 10 minutes, about 11 minutes, about 12 minutes, about 13 minutes, about 14

minutes, about 15 minutes, about 20 minutes, about 30 minutes, about 60 minutes, about 90 minutes, or about 2 hours of injury or suspected injury to the head. In some embodiments, the onset of the presence of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, appears within about 0 minutes, about 1 minute, about 2 minutes, about 3 minutes, about 4 minutes, about 5 minutes, about 6 minutes, about 7 minutes, about 8 minutes, about 9 minutes, about 10 minutes, about 11 minutes, about 12 minutes, about 13 minutes, about 14 minutes, about 15 minutes, about 20 minutes, about 30 minutes, about 60 minutes, about 90 minutes, or about 2 hours after injury to the head.

[0157] In some embodiments, the subject has received a CT scan before or after the assay is performed. In some embodiments, the subject is suspected as having a traumatic brain injury based on the CT scan. In some embodiments, the reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, is correlated with positive head CT scan.

[0158] Generally, a reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, can be employed as a benchmark against which to assess results obtained upon assaying a test sample for UCH-L1. Generally, in making such a comparison, the reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, is obtained by running a particular assay a sufficient number of times and under appropriate conditions such that a linkage or association of analyte presence, amount or concentration with a particular stage or endpoint of TBI or with particular indicia can be made. Typically, the reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, is obtained with assays of reference subjects (or populations of subjects). The early biomarker, such as UCH-L1, GFAP, or a combination thereof, measured can include fragments thereof, degradation products thereof, and/or enzymatic cleavage products thereof.

[0159] In some embodiments, the reference level for UCH-L1 is about 73.5 pg/mL. In some embodiments, the reference level for UCH-L1 is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 4.0 fold, 4.5 fold, or 5.0 fold greater than about 73.5 pg/mL.

[0160] In some embodiments, the reference level for UCH-L1 is about 88.2 pg/mL. In some embodiments, the reference level for UCH-L1 is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 4.0 fold, 4.5 fold, or 5.0 fold greater than 88.2 pg/mL.

[0161] In some embodiments, the reference level for UCH-L1 is about 371 pg/mL. In some embodiments, the reference level for UCH-L1 is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 4.0 fold, 4.5 fold, or 5.0 fold greater than about 371 pg/mL.

[0162] In some embodiments, the reference level for GFAP is about 9.0 pg/mL. In some embodiments, the reference level for GFAP is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 4.0 fold, 4.5 fold, or 5.0 fold greater than about 9.0 pg/mL.

[0163] In some embodiments, the reference level for GFAP is about11.7 pg/mL. In some embodiments, the reference level for GFAP is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 4.0 fold, 4.5 fold, or 5.0 fold greater than about 11.7 pg/mL.

[0164] In some embodiments, the reference level for GFAP is about 371 pg/mL. In some embodiments, the reference level for GFAP is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 4.0 fold, 4.5 fold, or 5.0 fold greater than about 371 pg/mL.

[0165] In some embodiments, the reference level can be any one of the values or range of values shown in Tables 2 and/or 3.

[0166] In some embodiments, the sample is taken (a) within about 10 minutes after the suspected injury and the reference level of UCH-L1 is at least about 1669 pg/mL; (b) within about 12 minutes after the suspected injury and the reference level of UCH-L1 is at least about 3285 pg/mL; (c) within about 20 minutes after the suspected injury and the reference level of UCH-L1 is at least about 2919 pg/mL; (d) within about 10 minutes after the suspected injury and the reference level of GFAP is at least about 108 pg/mL; (e) within about 12 minutes after the suspected injury and the reference level of GFAP is at least about 54 pg/mL; (f) within about 20 minutes after the suspected injury and the reference level of GFAP is at least about 1809 pg/mL; (g) within about 10 minutes after the suspected injury and the reference level of UCH-L1 is at least about 1669 pg/mL and the reference level of GFAP is at least about 108 pg/mL; (h) within about 12 minutes after the suspected injury and the reference level of UCH-L1 is at least about 3285 pg/mL and the reference level of GFAP is at least about 54 pg/mL; or (i) within about 20 minutes after the suspected injury and the reference level of UCH-L1 is at least about 2919 pg/mL and the reference level of GFAP is at least about 1809 pg/mL.

[0167] In some embodiments, the reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, is determined by an assay having a sensitivity of between at least about 65% to about 100% and a specificity of between at least about 30% to about 100%. In some embodiments, the sensitivity is between at least about 65% to about 100%, between at least about 65% to at least about 99%, between at least about 65% to at least about 95%, between at least about 65% to at least about 90%, between at least about 65% to at least about 85%, between at least about 65% to at least about 80%, between at least about 65% to at least about 75%, between at least about 65% to at least about 70%, between at least about

75% to about 100%, between at least about 75% to at least about 99%, between at least about 75% to at least about 95%, between at least about 75% to at least about 90%, between at least about 75% to at least about 85%, between at least about 75% to at least about 80%, between at least about 85% to about 100%, between at least about 85% to at least about 99%, between at least about 85% to at least about 95%, between at least about 85% to at least about 90%, between at least about 95% to about 100%, or between at least about 95% to at least about 99%. In some embodiments, the sensitivity is at least about 65.0%, at least about 70.0%, at least about 75.0%, at least about 80.0%, at least about 85.0%, at least about 87.5%, at least about 90.0%, at least about 95.0%, at least about 99.0%, at least about 99.1%, at least about 99.2%, at least about 99.3%, at least about 99.4%, at least about 99.5%, at least about 99.6%, at least about 99.7%, at least about 99.8%, at least about 99.9%, or at least about 100.0%.

[0168] In some embodiments, the specificity is between at least about 30% to about 100%, between at least about 30% to about 99%, between at least about 30% to about 95%, between at least about 30% to about 90%, between at least about 30% to about 85%, between at least about 30% to about 80%, between at least about 30% to about 75%, between at least about 30% to about 70%, between at least about 30% to about 60%, between at least about 30% to about 50%, between at least about 40% to about 100%, between at least about 40% to about 99%, between at least about 40% to about 95%, between at least about 40% to about 90%, between at least about 40% to about 85%, between at least about 40% to about 80%, between at least about 40% to about 75%, between at least about 40% to about 70%, between at least about 40% to about 60%, between at least about 40% to about 50%, between at least about 50% to about 100%, between at least about 50% to about 99%, between at least about 50% to about 95%, between at least about 50% to about 90%, between at least about 50% to about 85%, between at least about 50% to about 80%, between at least about 50% to about 75%, between at least about 50% to about 70%, between at least about 50% to about 60%, between at least about 60% to about 100%, between at least about 60% to about 99%, between at least about 60% to about 95%, between at least about 60% to about 90%, between at least about 60% to about 85%, between at least about 60% to about 80%, between at least about 60% to about 75%, between at least about 60% to about 70%, between at least about 70% to about 100%, between at least about 70% to about 99%, between at least about 70% to about 95%, between at least about 70% to about 90%, between at least about 70% to about 85%, between at least about 70% to about 80%, between at least about 70% to about 75%, between at least about 80% to about 100%, between at least about 80% to about 99%, between at least about 80% to about 95%, between at least about 80% to about 90%, between at least about 80% to

about 85%, between at least about 90% to about 100%, between at least about 90% to about 99%, between at least about 90% to about 95%, between at least about 95% to about 99%, or between at least about 95% to about 100. In some embodiments, the specificity is at least about 30.0%, at least about 31.0%, at least about 32.0%, at least about 33.0%, at least about 34.0%, at least about 35.0%, at least about 36.0%, at least about 37.0%, at least about 38.0%, at least about 39.0%, at least about 40.0%, at least about 45.0%, at least about 50.0%, at least about 55.0%, at least about 60.0%, at least about 65.0%, at least about 70.0%, at least about 75.0%, at least about 80.0%, at least about 85.0%, at least about 90.0%, at least about 91.0%, at least about 92.0%, at least about 93.0%, at least about 94.0%, at least about 95.0%, at least about 96.0%, at least about 97.0%, at least about 98.0%, at least about 99.0%, at least about 99.1%, at least about 99.2%, at least about 99.3%, at least about 99.4%, at least about 99.5%, at least about 99.6%, at least about 99.7%, at least about 99.8%, at least about 99.9%, or at least about 100.0%. For example, the sensitivity is at least about 99% and the specificity is at least about 75%, the sensitivity is at least about 99% and the specificity is at least about 99%, or the sensitivity is at least about 100% and the specificity is at least about 100%.

[0169] In some embodiments, the reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, can be between at least about 5 pg/mL to about 3500 pg/mL. In some embodiments, the reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, can be between at least about 10 pg/mL to about 3500 pg/mL, between at least about 10 pg/mL to about 3000 pg/mL, between at least about 10 pg/mL to about 2500 pg/mL, between at least about 10 pg/mL to about 2000 pg/mL, between at least about 10 pg/mL to about 1500 pg/mL, between at least about 10 pg/mL to about 1000 pg/mL, between at least about 10 pg/mL to about 900 pg/mL, between at least about 10 pg/mL to about 800 pg/mL, between at least about 10 pg/mL to about 750 pg/mL, between at least about 10 pg/mL to about 700 pg/mL, between at least about 10 pg/mL to about 600 pg/mL, between at least about 10 pg/mL to about 500 pg/mL, between at least about 10 pg/mL to about 400 pg/mL, between at least about 10 pg/mL to about 300 pg/mL, between at least about 10 pg/mL to about 200 pg/mL, between at least about 50 pg/mL to about 3500 pg/mL, between at least about 50 pg/mL to about 3000 pg/mL, between at least about 50 pg/mL to about 2500 pg/mL, between at least about 50 pg/mL to about 2000 pg/mL, between at least about 50 pg/mL to about 1500 pg/mL, between at least about 50 pg/mL to about 1000 pg/mL, between at least about 50 pg/mL to about 900 pg/mL, between at least about 50 pg/mL to about 800 pg/mL, between at least about 50 pg/mL to about 750 pg/mL, between at least about 50 pg/mL to about 700 pg/mL, between at least about 50 pg/mL to about 600

pg/mL, between at least about 50 pg/mL to about 500 pg/mL, between at least about 50 pg/mL to about 400 pg/mL, between at least about 50 pg/mL to about 300 pg/mL, between at least about 50 pg/mL to about 200 pg/mL, between at least about 100 pg/mL to about 3500 pg/mL, between at least about 100 pg/mL to about 3000 pg/mL, between at least about 100 pg/mL to about 2500 pg/mL, between at least about 100 pg/mL to about 2000 pg/mL, between at least about 100 pg/mL to about 1500 pg/mL, between at least about 100 pg/mL to about 1000 pg/mL, between at least about 100 pg/mL to about 900 pg/mL, between at least about 100 pg/mL to about 800 pg/mL, between at least about 100 pg/mL to about 750 pg/mL, between at least about 100 pg/mL to about 700 pg/mL, between at least about 100 pg/mL to about 600 pg/mL, between at least about 100 pg/mL to about 500 pg/mL, between at least about 100 pg/mL to about 400 pg/mL, between at least about 100 pg/mL to about 300 pg/mL, between at least about 100 pg/mL to about 200 pg/mL, between at least about 150 pg/mL to about 3500 pg/mL, between at least about 150 pg/mL to about 3000 pg/mL, between at least about 150 pg/mL to about 2500 pg/mL, between at least about 150 pg/mL to about 2000 pg/mL, between at least about 150 pg/mL to about 1500 pg/mL, between at least about 150 pg/mL to about 1000 pg/mL, between at least about 150 pg/mL to about 900 pg/mL, between at least about 150 pg/mL to about 800 pg/mL, between at least about 150 pg/mL to about 750 pg/mL, between at least about 150 pg/mL to about 700 pg/mL, between at least about 150 pg/mL to about 600 pg/mL, between at least about 150 pg/mL to about 500 pg/mL, between at least about 150 pg/mL to about 400 pg/mL, between at least about 150 pg/mL to about 300 pg/mL, between at least about 150 pg/mL to about 200 pg/mL, between at least about 200 pg/mL to about 3500 pg/mL, between at least about 200 pg/mL to about 3000 pg/mL, between at least about 200 pg/mL to about 2500 pg/mL, between at least about 200 pg/mL to about 2000 pg/mL, between at least about 200 pg/mL to about 1500 pg/mL, between at least about 200 pg/mL to about 1000 pg/mL, between at least about 200 pg/mL to about 900 pg/mL, between at least about 200 pg/mL to about 800 pg/mL, between at least about 200 pg/mL to about 750 pg/mL, between at least about 200 pg/mL to about 700 pg/mL, between at least about 200 pg/mL to about 600 pg/mL, between at least about 200 pg/mL to about 500 pg/mL, between at least about 200 pg/mL to about 400 pg/mL, between at least about 200 pg/mL to about 300 pg/mL, between at least about 300 pg/mL to about 3500 pg/mL, between at least about 300 pg/mL to about 3000 pg/mL, between at least about 300 pg/mL to about 2500 pg/mL, between at least about 300 pg/mL to about 2000 pg/mL, between at least about 300 pg/mL to about 1500 pg/mL, between at least about 300 pg/mL to about 1000 pg/mL, between at least about 300 pg/mL to about 900 pg/mL, between at least about 300 pg/mL to

about 800 pg/mL, between at least about 300 pg/mL to about 750 pg/mL, between at least about 300 pg/mL to about 700 pg/mL, between at least about 300 pg/mL to about 600 pg/mL, between at least about 300 pg/mL to about 500 pg/mL, between at least about 300 pg/mL to about 400 pg/mL, between at least about 400 pg/mL to about 3500 pg/mL, between at least about 400 pg/mL to about 3000 pg/mL, between at least about 400 pg/mL to about 2500 pg/mL, between at least about 400 pg/mL to about 2000 pg/mL, between at least about 400 pg/mL to about 1500 pg/mL, between at least about 400 pg/mL to about 1000 pg/mL, between at least about 400 pg/mL to about 900 pg/mL, between at least about 400 pg/mL to about 800 pg/mL, between at least about 400 pg/mL to about 750 pg/mL, between at least about 400 pg/mL to about 700 pg/mL, between at least about 400 pg/mL to about 600 pg/mL, between at least about 400 pg/mL to about 500 pg/mL, between at least about 500 pg/mL to about 3500 pg/mL, between at least about 500 pg/mL to about 3000 pg/mL, between at least about 500 pg/mL to about 2500 pg/mL, between at least about 500 pg/mL to about 2000 pg/mL, between at least about 500 pg/mL to about 1500 pg/mL, between at least about 500 pg/mL to about 1000 pg/mL, between at least about 500 pg/mL to about 900 pg/mL, between at least about 500 pg/mL to about 800 pg/mL, between at least about 500 pg/mL to about 750 pg/mL, between at least about 500 pg/mL to about 700 pg/mL, or between at least about 500 pg/mL to about 600 pg/mL. For example, the reference level of the early biomarker, such as UCH-L1, GFAP, or a combination thereof, can be at least about 5 pg/mL, can be at least about 10 pg/mL, at least about 20 pg/mL, at least about 30 pg/mL, at least about 40 pg/mL, at least about 50 pg/mL, at least about 55 pg/mL, at least about 60 pg/mL, at least about 65 pg/mL, at least about 70 pg/mL, at least about 75 pg/mL, at least about 80 pg/mL, at least about 85 pg/mL, at least about 90 pg/mL, at least about 95 pg/mL, at least about 100 pg/mL, at least about 150 pg/mL, at least about 200 pg/mL, at least about 250 pg/mL, at least about 300 pg/mL, at least about 350 pg/mL, at least about 400 pg/mL, at least about 450 pg/mL, at least about 500 pg/mL, at least about 550 pg/mL, at least about 600 pg/mL, at least abo700 pg/mL, at least about 800 pg/mL, at least about 900 pg/mL, at least about 1000 pg/mL, at least about 1500 pg/mL, at least about 2000 pg/mL, at least about 2500 pg/mL, at least about 3000 pg/mL, or at least about 3500 pg/mL.

[0170] In some embodiments, the method further includes treating the human subject with a traumatic brain injury treatment and/or monitoring the human subject, as described below.

[0171] The nature of the assay employed in the methods described herein is not critical and the test can be any assay known in the art such as, for example, immunoassays, protein immunoprecipitation, immunoelectrophoresis, Western blot, or protein immunostaining, or

spectrometry methods, such as high-performance liquid chromatography (HPLC) or liquid chromatography–mass spectrometry (LC/MS). Also, the assay can be employed in clinical chemistry format such as would be known by one skilled in the art. Such assays are described in further detail herein in Sections 5-9.

4. Treatment and Monitoring of Subjects Suffering from Traumatic Brain Injury

[0172] The subject identified or assessed in the methods described above as having traumatic brain injury, such as mild traumatic brain injury or moderate to severe traumatic brain injury, may be treated or monitored. In some embodiments, the method further includes treating the human subject assessed as having traumatic brain injury with a traumatic brain injury treatment, such as any treatments known in the art. For example, treatment of traumatic brain injury can take a variety of forms depending on the severity of the injury to the head. For example, for subjects suffering from mild TBI, the treatment may include one or more of rest, abstaining for physical activities, such as sports, avoiding light or wearing sunglasses when out in the light, medication for relief of a headache or migraine, anti-nausea medication, etc. Treatment for patients suffering from severe TBI might include administration of one or more appropriate medications (such as, for example, diuretics, anti-convulsant medications, medications to sedate and put an individual in a drug-induced coma, or other pharmaceutical or biopharmaceutical medications (either known or developed in the future for treatment of TBI), one or more surgical procedures (such as, for example, removal of a hematoma, repairing a skull fracture, decompressive craniectomy, etc.) and one or more therapies (such as, for example one or more rehabilitation, cognitive behavioral therapy, anger management, counseling psychology, etc.). In some embodiments, the method further includes monitoring the human subject assessed as having traumatic brain injury (*e.g.*, mild or moderate to severe traumatic brain injury, or mild, moderate, severe, or moderate to severetraumatic brain injury). In some embodiments, a subject identified as having traumatic brain injury, such as mild traumatic brain injury or severe traumatic brain injury or mild traumatic brain injury, moderate traumatic brain injury, severe traumatic brain injury, or moderate to severe traumatic brain injury may be monitored with CT scan or MRI.

5. Methods for Measuring the Level of UCH-L1

[0173] In the methods described above, UCH-L1 levels can be measured by any means, such as antibody dependent methods, such as immunoassays, protein immunoprecipitation, immunoelectrophoresis, chemical analysis, SDS-PAGE and Western blot analysis, protein

immunostaining, electrophoresis analysis, a protein assay, a competitive binding assay, a functional protein assay, or chromatography or spectrometry methods, such as high-performance liquid chromatography (HPLC) or liquid chromatography–mass spectrometry (LC/MS). Also, the assay can be employed in clinical chemistry format such as would be known by one skilled in the art.

[0174] In some embodiments, measuring the level of UCH-L1 includes contacting the sample with a first specific binding member and second specific binding member. In some embodiments the first specific binding member is a capture antibody and the second specific binding member is a detection antibody. In some embodiments, measuring the level of UCH-L1 includes contacting the sample, either simultaneously or sequentially, in any order: (1) a capture antibody (e.g., UCH-L1-capture antibody), which binds to an epitope on UCH-L1 or UCH-L1 fragment to form a capture antibody-UCH-L1 antigen complex (e.g., UCH-L1-capture antibody-UCH-L1 antigen complex), and (2) a detection antibody (e.g., UCH-L1-detection antibody), which includes a detectable label and binds to an epitope on UCH-L1 that is not bound by the capture antibody, to form a UCH-L1 antigen-detection antibody complex (e.g., UCH-L1 antigen-UCH-L1-detection antibody complex), such that a capture antibody-UCH-L1 antigen-detection antibody complex (e.g., UCH-L1-capture antibody-UCH-L1 antigen-UCH-L1-detection antibody complex) is formed, and measuring the amount or concentration of UCH-L1 in the sample based on the signal generated by the detectable label in the capture antibody-UCH-L1 antigen-detection antibody complex.

[0175] In some embodiments, the first specific binding member is immobilized on a solid support. In some embodiments, the second specific binding member is immobilized on a solid support. In some embodiments, the first specific binding member is a UCH-L1 antibody as described below.

[0176] In some embodiments, the sample is diluted or undiluted. The sample can be from about 1 to about 25 microliters, about 1 to about 24 microliters, about 1 to about 23 microliters, about 1 to about 22 microliters, about 1 to about 21 microliters, about 1 to about 20 microliters, about 1 to about 18 microliters, about 1 to about 17 microliters, about 1 to about 16 microliters, about 15 microliters or about 1 microliter, about 2 microliters, about 3 microliters, about 4 microliters, about 5 microliters, about 6 microliters, about 7 microliters, about 8 microliters, about 9 microliters, about 10 microliters, about 11 microliters, about 12 microliters, about 13 microliters, about 14 microliters, about 15 microliters, about 16 microliters, about 17 microliters, about 18 microliters, about 19 microliters, about 20 microliters, about 21 microliters, about 22 microliters, about 23 microliters, about 24

microliters or about 25 microliters. In some embodiments, the sample is from about 1 to about 150 microliters or less or from about 1 to about 25 microliters or less.

[0177] Some instruments (such as, for example the Abbott Laboratories instrument ARCHITECT®, and other core laboratory instruments) other than a point-of-care device may be capable of measuring levels of UCH-L1 in a sample higher or greater than 25,000 pg/mL.

[0178] Other methods of detection include the use of or can be adapted for use on a nanopore device or nanowell device. Examples of nanopore devices are described in International Patent Publication No. WO 2016/161402, which is hereby incorporated by reference in its entirety. Examples of nanowell device are described in International Patent Publication No. WO 2016/161400, which is hereby incorporated by reference in its entirety

6. UCH-L1 Antibodies

[0179] The methods described herein may use an isolated antibody that specifically binds to ubiquitin carboxy-terminal hydrolase L1 ("UCH-L1") (or fragments thereof), referred to as "UCH-L1 antibody." The UCH-L1 antibodies can be used to assess the UCH-L1 status as a measure of traumatic brain injury, detect the presence of UCH-L1 in a sample, quantify the amount of UCH-L1 present in a sample, or detect the presence of and quantify the amount of UCH-L1 in a sample.

a. Ubiquitin Carboxy-Terminal Hydrolase L1 (UCH-L1)

[0180] Ubiquitin carboxy-terminal hydrolase L1 ("UCH-L1"), which is also known as "ubiquitin C-terminal hydrolase," is a deubiquitinating enzyme. UCH-L1 is a member of a gene family whose products hydrolyze small C-terminal adducts of ubiquitin to generate the ubiquitin monomer. Expression of UCH-L1 is highly specific to neurons and to cells of the diffuse neuroendocrine system and their tumors. It is abundantly present in all neurons (accounts for 1-2% of total brain protein), expressed specifically in neurons and testis/ovary. The catalytic triad of UCH-L1 contains a cysteine at position 90, an aspartate at position 176, and a histidine at position 161 that are responsible for its hydrolase activity.

[0181] Human UCH-L1 may have the following amino acid sequence:

[0182] MQLKPMEINPEMLNKVLSRLGVAGQWRFVDVLGLEEESLGSVPAPACALL LLFPLTAQHENFRKKQIEELKGQEVSPKVYFMKQTIGNSCGTIGLIHAVANNQDKLGF EDGSVLKQFLSETEKMSPEDRAKCFEKNEAIQAAHDAVAQEGQCRVDDKVNFHFIL FNNVDGHLYELDGRMPFPVNHGASSEDTLLKDAAKVCREFTEREQGEVRFSAVALC KAA (SEQ ID NO: 1).

[0183] The human UCH-L1 may be a fragment or variant of SEQ ID NO: 1. The fragment of UCH-L1 may be between 5 and 225 amino acids, between 10 and 225 amino acids, between 50 and 225 amino acids, between 60 and 225 amino acids, between 65 and 225 amino acids, between 100 and 225 amino acids, between 150 and 225 amino acids, between 100 and 175 amino acids, or between 175 and 225 amino acids in length. The fragment may comprise a contiguous number of amino acids from SEQ ID NO: 1.

b. UCH-L1-Recognizing Antibody

[0184] The antibody is an antibody that binds to UCH-L1, a fragment thereof, an epitope of UCH-L1, or a variant thereof. The antibody may be a fragment of the anti-UCH-L1 antibody or a variant or a derivative thereof. The antibody may be a polyclonal or monoclonal antibody. The antibody may be a chimeric antibody, a single chain antibody, an affinity matured antibody, a human antibody, a humanized antibody, a fully human antibody or an antibody fragment, such as a Fab fragment, or a mixture thereof. Antibody fragments or derivatives may comprise $F(ab')_2$, Fv or scFv fragments. The antibody derivatives can be produced by peptidomimetics. Further, techniques described for the production of single chain antibodies can be adapted to produce single chain antibodies.

[0185] The anti-UCH-L1 antibodies may be a chimeric anti-UCH-L1 or humanized anti-UCH-L1 antibody. In one embodiment, both the humanized antibody and chimeric antibody are monovalent. In one embodiment, both the humanized antibody and chimeric antibody comprise a single Fab region linked to an Fc region.

[0186] Human antibodies may be derived from phage-display technology or from transgenic mice that express human immunoglobulin genes. The human antibody may be generated as a result of a human in vivo immune response and isolated. See, for example, Funaro *et al.*, *BMC Biotechnology*, 2008(8):85. Therefore, the antibody may be a product of the human and not animal repertoire. Because it is of human origin, the risks of reactivity against self-antigens may be minimized. Alternatively, standard yeast display libraries and display technologies may be used to select and isolate human anti-UCH-L1 antibodies. For example, libraries of naïve human single chain variable fragments (scFv) may be used to select human anti-UCH-L1 antibodies. Transgenic animals may be used to express human antibodies.

[0187] Humanized antibodies may be antibody molecules from non-human species antibody that binds the desired antigen having one or more complementarity determining

regions (CDRs) from the non-human species and framework regions from a human immunoglobulin molecule.

[0188] The antibody is distinguishable from known antibodies in that it possesses different biological function(s) than those known in the art.

(1) Epitope

[0189] The antibody may immunospecifically bind to UCH-L1 (SEQ ID NO: 1), a fragment thereof, or a variant thereof. The antibody may immunospecifically recognize and bind at least three amino acids, at least four amino acids, at least five amino acids, at least six amino acids, at least seven amino acids, at least eight amino acids, at least nine amino acids, or at least ten amino acids within an epitope region. The antibody may immunospecifically recognize and bind to an epitope that has at least three contiguous amino acids, at least four contiguous amino acids, at least five contiguous amino acids, at least six contiguous amino acids, at least seven contiguous amino acids, at least eight contiguous amino acids, at least nine contiguous amino acids, or at least ten contiguous amino acids of an epitope region.

c. Antibody Preparation/Production

[0190] Antibodies may be prepared by any of a variety of techniques, including those well known to those skilled in the art. In general, antibodies can be produced by cell culture techniques, including the generation of monoclonal antibodies via conventional techniques, or via transfection of antibody genes, heavy chains, and/or light chains into suitable bacterial or mammalian cell hosts, in order to allow for the production of antibodies, wherein the antibodies may be recombinant. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, e.g., electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. Although it is possible to express the antibodies in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells is preferable, and most preferable in mammalian host cells, because such eukaryotic cells (and in particular mammalian cells) are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody.

[0191] Exemplary mammalian host cells for expressing the recombinant antibodies include Chinese Hamster Ovary (CHO cells) (including dhfr-CHO cells, described in Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77: 4216-4220 (1980)), used with a DHFR selectable marker, *e.g.*, as described in Kaufman and Sharp, *J. Mol. Biol.*, 159: 601-621 (1982), NS0 myeloma cells, COS cells, and SP2 cells. When recombinant expression vectors encoding

antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

[0192] Host cells can also be used to produce functional antibody fragments, such as Fab fragments or scFv molecules. It will be understood that variations on the above procedure may be performed. For example, it may be desirable to transfect a host cell with DNA encoding functional fragments of either the light chain and/or the heavy chain of an antibody. Recombinant DNA technology may also be used to remove some, or all, of the DNA encoding either or both of the light and heavy chains that is not necessary for binding to the antigens of interest. The molecules expressed from such truncated DNA molecules are also encompassed by the antibodies. In addition, bifunctional antibodies may be produced in which one heavy and one light chain are an antibody (i.e., binds human UCH-L1) and the other heavy and light chain are specific for an antigen other than human UCH-L1 by crosslinking an antibody to a second antibody by standard chemical crosslinking methods.

[0193] In a preferred system for recombinant expression of an antibody, or antigen-binding portion thereof, a recombinant expression vector encoding both the antibody heavy chain and the antibody light chain is introduced into dhfr-CHO cells by calcium phosphate-mediated transfection. Within the recombinant expression vector, the antibody heavy and light chain genes are each operatively linked to CMV enhancer/AdMLP promoter regulatory elements to drive high levels of transcription of the genes. The recombinant expression vector also carries a DHFR gene, which allows for selection of CHO cells that have been transfected with the vector using methotrexate selection/amplification. The selected transformant host cells are cultured to allow for expression of the antibody heavy and light chains and intact antibody is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture the host cells, and recover the antibody from the culture medium. Still further, the method of synthesizing a recombinant antibody may be by culturing a host cell in a suitable culture medium until a recombinant antibody is synthesized. The method can further comprise isolating the recombinant antibody from the culture medium.

[0194] Methods of preparing monoclonal antibodies involve the preparation of immortal cell lines capable of producing antibodies having the desired specificity. Such cell lines may

be produced from spleen cells obtained from an immunized animal. The animal may be immunized with UCH-L1 or a fragment and/or variant thereof. The peptide used to immunize the animal may comprise amino acids encoding human Fc, for example the fragment crystallizable region or tail region of human antibody. The spleen cells may then be immortalized by, for example, fusion with a myeloma cell fusion partner. A variety of fusion techniques may be employed. For example, the spleen cells and myeloma cells may be combined with a nonionic detergent for a few minutes and then plated at low density on a selective medium that supports that growth of hybrid cells, but not myeloma cells. One such technique uses hypoxanthine, aminopterin, thymidine (HAT) selection. Another technique includes electrofusion. After a sufficient time, usually about 1 to 2 weeks, colonies of hybrids are observed. Single colonies are selected and their culture supernatants tested for binding activity against the polypeptide. Hybridomas having high reactivity and specificity may be used.

[0195] Monoclonal antibodies may be isolated from the supernatants of growing hybridoma colonies. In addition, various techniques may be employed to enhance the yield, such as injection of the hybridoma cell line into the peritoneal cavity of a suitable vertebrate host, such as a mouse. Monoclonal antibodies may then be harvested from the ascites fluid or the blood. Contaminants may be removed from the antibodies by conventional techniques, such as chromatography, gel filtration, precipitation, and extraction. Affinity chromatography is an example of a method that can be used in a process to purify the antibodies.

[0196] The proteolytic enzyme papain preferentially cleaves IgG molecules to yield several fragments, two of which (the F(ab) fragments) each comprise a covalent heterodimer that includes an intact antigen-binding site. The enzyme pepsin is able to cleave IgG molecules to provide several fragments, including the $F(ab')_2$ fragment, which comprises both antigen-binding sites.

[0197] The Fv fragment can be produced by preferential proteolytic cleavage of an IgM, and on rare occasions IgG or IgA immunoglobulin molecules. The Fv fragment may be derived using recombinant techniques. The Fv fragment includes a non-covalent VH::VL heterodimer including an antigen-binding site that retains much of the antigen recognition and binding capabilities of the native antibody molecule.

[0198] The antibody, antibody fragment, or derivative may comprise a heavy chain and a light chain complementarity determining region ("CDR") set, respectively interposed between a heavy chain and a light chain framework ("FR") set which provide support to the

CDRs and define the spatial relationship of the CDRs relative to each other. The CDR set may contain three hypervariable regions of a heavy or light chain V region.

[0199] Other suitable methods of producing or isolating antibodies of the requisite specificity can be used, including, but not limited to, methods that select recombinant antibody from a peptide or protein library (e.g., but not limited to, a bacteriophage, ribosome, oligonucleotide, RNA, cDNA, yeast or the like, display library); e.g., as available from various commercial vendors such as Cambridge Antibody Technologies (Cambridgeshire, UK), MorphoSys (Martinsreid/Planegg, Del.), Biovation (Aberdeen, Scotland, UK) BioInvent (Lund, Sweden), using methods known in the art. See U.S. Patent Nos. 4,704,692; 5,723,323; 5,763,192; 5,814,476; 5,817,483; 5,824,514; 5,976,862. Alternative methods rely upon immunization of transgenic animals (*e.g.*, SCID mice, Nguyen *et al.* (1997) *Microbiol. Immunol.* 41:901-907; Sandhu *et al.* (1996) *Crit. Rev. Biotechnol.* 16:95-118; Eren *et al.* (1998) *Immunol.* 93:154-161) that are capable of producing a repertoire of human antibodies, as known in the art and/or as described herein. Such techniques, include, but are not limited to, ribosome display (Hanes *et al.* (1997) *Proc. Natl. Acad. Sci. USA*, 94:4937-4942; Hanes *et al.* (1998) *Proc. Natl. Acad. Sci. USA*, 95:14130-14135); single cell antibody producing technologies (e.g., selected lymphocyte antibody method ("SLAM") (U.S. Patent No. 5,627,052, Wen *et al.* (1987) *J. Immunol.* 17:887-892; Babcook *et al.* (1996) *Proc. Natl. Acad. Sci. USA* 93:7843-7848); gel microdroplet and flow cytometry (Powell *et al.* (1990) Biotechnol. 8:333-337; One Cell Systems, (Cambridge, Mass).; Gray *et al.* (1995) J. Imm. Meth. 182:155-163; Kenny *et al.* (1995) Bio/Technol. 13:787-790); B-cell selection (Steenbakkers *et al.* (1994) *Molec. Biol. Reports* 19:125-134 (1994)).

[0200] An affinity matured antibody may be produced by any one of a number of procedures that are known in the art. For example, see Marks *et al.*, *BioTechnology*, 10: 779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by Barbas *et al.*, *Proc. Nat. Acad. Sci. USA*, 91: 3809-3813 (1994); Schier *et al.*, Gene, 169: 147-155 (1995); Yelton *et al.*, *J. Immunol.*, 155: 1994-2004 (1995); Jackson *et al.*, *J. Immunol.*, 154(7): 3310-3319 (1995); Hawkins *et al*, *J. Mol. Biol.*, 226: 889-896 (1992). Selective mutation at selective mutagenesis positions and at contact or hypermutation positions with an activity enhancing amino acid residue is described in U.S. Patent No. 6,914,128 B1.

[0201] Antibody variants can also be prepared using delivering a polynucleotide encoding an antibody to a suitable host such as to provide transgenic animals or mammals, such as goats, cows, horses, sheep, and the like, that produce such antibodies in their milk. These

methods are known in the art and are described for example in U.S. Patent Nos. 5,827,690; 5,849,992; 4,873,316; 5,849,992; 5,994,616; 5,565,362; and 5,304,489.

[0202] Antibody variants also can be prepared by delivering a polynucleotide to provide transgenic plants and cultured plant cells (e.g., but not limited to tobacco, maize, and duckweed) that produce such antibodies, specified portions or variants in the plant parts or in cells cultured therefrom. For example, Cramer *et al.* (1999) *Curr. Top. Microbiol. Immunol.* 240:95-118 and references cited therein, describe the production of transgenic tobacco leaves expressing large amounts of recombinant proteins, *e.g.*, using an inducible promoter. Transgenic maize have been used to express mammalian proteins at commercial production levels, with biological activities equivalent to those produced in other recombinant systems or purified from natural sources. See, e.g., Hood *et al.*, *Adv. Exp. Med. Biol.* (1999) 464:127-147 and references cited therein. Antibody variants have also been produced in large amounts from transgenic plant seeds including antibody fragments, such as single chain antibodies (scFv's), including tobacco seeds and potato tubers. See, e.g., Conrad *et al.* (1998) *Plant Mol. Biol.* 38:101-109 and reference cited therein. Thus, antibodies can also be produced using transgenic plants, according to known methods.

[0203] Antibody derivatives can be produced, for example, by adding exogenous sequences to modify immunogenicity or reduce, enhance or modify binding, affinity, on-rate, off-rate, avidity, specificity, half-life, or any other suitable characteristic. Generally, part or all of the non-human or human CDR sequences are maintained while the non-human sequences of the variable and constant regions are replaced with human or other amino acids.

[0204] Small antibody fragments may be diabodies having two antigen-binding sites, wherein fragments comprise a heavy chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain (VH VL). See for example, EP 404,097; WO 93/11161; and Hollinger *et al.*, (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448. By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. See also, U.S. Patent No. 6,632,926 to Chen *et al.* which is hereby incorporated by reference in its entirety and discloses antibody variants that have one or more amino acids inserted into a hypervariable region of the parent antibody and a binding affinity for a target antigen which is at least about two fold stronger than the binding affinity of the parent antibody for the antigen.

[0205] The antibody may be a linear antibody. The procedure for making a linear antibody is known in the art and described in Zapata *et al.*, (1995) *Protein Eng.* 8(10):1057-

1062. Briefly, these antibodies comprise a pair of tandem Fd segments (VH-CH1-VH-CH1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

[0206] The antibodies may be recovered and purified from recombinant cell cultures by known methods including, but not limited to, protein A purification, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. High performance liquid chromatography ("HPLC") can also be used for purification.

[0207] It may be useful to detectably label the antibody. Methods for conjugating antibodies to these agents are known in the art. For the purpose of illustration only, antibodies can be labeled with a detectable moiety such as a radioactive atom, a chromophore, a fluorophore, or the like. Such labeled antibodies can be used for diagnostic techniques, either in vivo, or in an isolated test sample. They can be linked to a cytokine, to a ligand, to another antibody. Suitable agents for coupling to antibodies to achieve an anti-tumor effect include cytokines, such as interleukin 2 (IL-2) and Tumor Necrosis Factor (TNF); photosensitizers, for use in photodynamic therapy, including aluminum (III) phthalocyanine tetrasulfonate, hematoporphyrin, and phthalocyanine; radionuclides, such as iodine-131 (131I), yttrium-90 (90Y), bismuth-212 (212Bi), bismuth-213 (213Bi), technetium-99m (99mTc), rhenium-186 (186Re), and rhenium-188 (188Re); antibiotics, such as doxorubicin, adriamycin, daunorubicin, methotrexate, daunomycin, neocarzinostatin, and carboplatin; bacterial, plant, and other toxins, such as diphtheria toxin, pseudomonas exotoxin A, staphylococcal enterotoxin A, abrin-A toxin, ricin A (deglycosylated ricin A and native ricin A), TGF-alpha toxin, cytotoxin from chinese cobra (naja naja atra), and gelonin (a plant toxin); ribosome inactivating proteins from plants, bacteria and fungi, such as restrictocin (a ribosome inactivating protein produced by Aspergillus restrictus), saporin (a ribosome inactivating protein from Saponaria officinalis), and RNase; tyrosine kinase inhibitors; ly207702 (a difluorinated purine nucleoside); liposomes containing anti cystic agents (*e.g.*, antisense oligonucleotides, plasmids which encode for toxins, methotrexate, etc.); and other antibodies or antibody fragments, such as F(ab).

[0208] Antibody production via the use of hybridoma technology, the selected lymphocyte antibody method (SLAM), transgenic animals, and recombinant antibody libraries is described in more detail below.

(1) Anti-UCH-L1 Monoclonal Antibodies Using Hybridoma Technology

[0209] Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow *et al.*, Antibodies: *A Laboratory Manual*, second edition, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1988); Hammerling, *et al.*, *In Monoclonal Antibodies and T-Cell Hybridomas*, (Elsevier, N.Y., 1981). It is also noted that the term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. The term "monoclonal antibody" refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced.

[0210] Methods of generating monoclonal antibodies as well as antibodies produced by the method may comprise culturing a hybridoma cell secreting an antibody of the invention wherein, preferably, the hybridoma is generated by fusing splenocytes isolated from an animal, *e.g.*, a rat or a mouse, immunized with UCH-L1 with myeloma cells and then screening the hybridomas resulting from the fusion for hybridoma clones that secrete an antibody able to bind a polypeptide of the invention. Briefly, rats can be immunized with a UCH-L1 antigen. In a preferred embodiment, the UCH-L1 antigen is administered with an adjuvant to stimulate the immune response. Such adjuvants include complete or incomplete Freund's adjuvant, RIBI (muramyl dipeptides) or ISCOM (immunostimulating complexes). Such adjuvants may protect the polypeptide from rapid dispersal by sequestering it in a local deposit, or they may contain substances that stimulate the host to secrete factors that are chemotactic for macrophages and other components of the immune system. Preferably, if a polypeptide is being administered, the immunization schedule will involve two or more administrations of the polypeptide, spread out over several weeks; however, a single administration of the polypeptide may also be used.

[0211] After immunization of an animal with a UCH-L1 antigen, antibodies and/or antibody-producing cells may be obtained from the animal. An anti-UCH-L1 antibody-containing serum is obtained from the animal by bleeding or sacrificing the animal. The serum may be used as it is obtained from the animal, an immunoglobulin fraction may be obtained from the serum, or the anti-UCH-L1 antibodies may be purified from the serum. Serum or immunoglobulins obtained in this manner are polyclonal, thus having a heterogeneous array of properties.

[0212] Once an immune response is detected, *e.g.*, antibodies specific for the antigen UCH-L1 are detected in the rat serum, the rat spleen is harvested and splenocytes isolated. The splenocytes are then fused by well-known techniques to any suitable myeloma cells, for example, cells from cell line SP20 available from the American Type Culture Collection (ATCC, Manassas, Va., US). Hybridomas are selected and cloned by limited dilution. The hybridoma clones are then assayed by methods known in the art for cells that secrete antibodies capable of binding UCH-L1. Ascites fluid, which generally contains high levels of antibodies, can be generated by immunizing rats with positive hybridoma clones.

[0213] In another embodiment, antibody-producing immortalized hybridomas may be prepared from the immunized animal. After immunization, the animal is sacrificed and the splenic B cells are fused to immortalized myeloma cells as is well known in the art. See, *e.g.*, Harlow and Lane, supra. In a preferred embodiment, the myeloma cells do not secrete immunoglobulin polypeptides (a non-secretory cell line). After fusion and antibiotic selection, the hybridomas are screened using UCH-L1, or a portion thereof, or a cell expressing UCH-L1. In a preferred embodiment, the initial screening is performed using an enzyme-linked immunosorbent assay (ELISA) or a radioimmunoassay (RIA), preferably an ELISA. An example of ELISA screening is provided in PCT Publication No. WO 00/37504.

[0214] Anti-UCH-L1 antibody-producing hybridomas are selected, cloned, and further screened for desirable characteristics, including robust hybridoma growth, high antibody production, and desirable antibody characteristics. Hybridomas may be cultured and expanded in vivo in syngeneic animals, in animals that lack an immune system, *e.g.*, nude mice, or in cell culture in vitro. Methods of selecting, cloning and expanding hybridomas are well known to those of ordinary skill in the art.

[0215] In a preferred embodiment, hybridomas are rat hybridomas. In another embodiment, hybridomas are produced in a non-human, non-rat species such as mice, sheep, pigs, goats, cattle, or horses. In yet another preferred embodiment, the hybridomas are human hybridomas, in which a human non-secretory myeloma is fused with a human cell expressing an anti-UCH-L1 antibody.

[0216] Antibody fragments that recognize specific epitopes may be generated by known techniques. For example, Fab and $F(ab')_2$ fragments of the invention may be produced by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to produce two identical Fab fragments) or pepsin (to produce an $F(ab')_2$ fragment). A $F(ab')_2$ fragment of an IgG molecule retains the two antigen-binding sites of the larger ("parent") IgG molecule, including both light chains (containing the variable light chain and constant light

chain regions), the CH1 domains of the heavy chains, and a disulfide-forming hinge region of the parent IgG molecule. Accordingly, an $F(ab')_2$ fragment is still capable of crosslinking antigen molecules like the parent IgG molecule.

(2) Anti-UCH-L1 Monoclonal Antibodies Using SLAM

[0217] In another aspect of the invention, recombinant antibodies are generated from single, isolated lymphocytes using a procedure referred to in the art as the selected lymphocyte antibody method (SLAM), as described in U.S. Patent No. 5,627,052; PCT Publication No. WO 92/02551; and Babcook *et al.*, *Proc. Natl. Acad. Sci. USA*, 93: 7843-7848 (1996). In this method, single cells secreting antibodies of interest, *e.g.*, lymphocytes derived from any one of the immunized animals are screened using an antigen-specific hemolytic plaque assay, wherein the antigen UCH-L1, a subunit of UCH-L1, or a fragment thereof, is coupled to sheep red blood cells using a linker, such as biotin, and used to identify single cells that secrete antibodies with specificity for UCH-L1. Following identification of antibody-secreting cells of interest, heavy- and light-chain variable region cDNAs are rescued from the cells by reverse transcriptase-PCR (RT-PCR) and these variable regions can then be expressed, in the context of appropriate immunoglobulin constant regions (*e.g.*, human constant regions), in mammalian host cells, such as COS or CHO cells. The host cells transfected with the amplified immunoglobulin sequences, derived from in vivo selected lymphocytes, can then undergo further analysis and selection in vitro, for example, by panning the transfected cells to isolate cells expressing antibodies to UCH-L1. The amplified immunoglobulin sequences further can be manipulated in vitro, such as by in vitro affinity maturation method. See, for example, PCT Publication No. WO 97/29131 and PCT Publication No. WO 00/56772.

(3) Anti-UCH-L1 Monoclonal Antibodies Using Transgenic Animals

[0218] In another embodiment of the invention, antibodies are produced by immunizing a non-human animal comprising some, or all, of the human immunoglobulin locus with a UCH-L1 antigen. In an embodiment, the non-human animal is a XENOMOUSE® transgenic mouse, an engineered mouse strain that comprises large fragments of the human immunoglobulin loci and is deficient in mouse antibody production. See, *e.g.*, Green *et al.*, *Nature Genetics*, 7: 13-21 (1994) and U.S. Patent Nos. 5,916,771; 5,939,598; 5,985,615; 5,998,209; 6,075,181; 6,091,001; 6,114,598; and 6,130,364. See also PCT Publication Nos. WO 91/10741; WO 94/02602; WO 96/34096; WO 96/33735; WO 98/16654; WO 98/24893; WO 98/50433; WO 99/45031; WO 99/53049; WO 00/09560; and WO 00/37504. The XENOMOUSE® transgenic mouse produces an adult-like human repertoire of fully human

antibodies, and generates antigen-specific human monoclonal antibodies. The XENOMOUSE® transgenic mouse contains approximately 80% of the human antibody repertoire through introduction of megabase sized, germline configuration YAC fragments of the human heavy chain loci and x light chain loci. See Mendez *et al.*, *Nature Genetics*, 15: 146-156 (1997), Green and Jakobovits, *J. Exp. Med.*, 188: 483-495 (1998), the disclosures of which are hereby incorporated by reference.

(4) Anti-UCH-L1 Monoclonal Antibodies Using Recombinant Antibody Libraries

[0219] In vitro methods also can be used to make the antibodies of the invention, wherein an antibody library is screened to identify an antibody having the desired UCH-L1 -binding specificity. Methods for such screening of recombinant antibody libraries are well known in the art and include methods described in, for example, U.S. Patent No. 5,223,409 (Ladner *et al.*); PCT Publication No. WO 92/18619 (Kang *et al.*); PCT Publication No. WO 91/17271 (Dower *et al.*); PCT Publication No. WO 92/20791 (Winter *et al.*); PCT Publication No. WO 92/15679 (Markland *et al.*); PCT Publication No. WO 93/01288 (Breitling *et al.*); PCT Publication No. WO 92/01047 (McCafferty *et al.*); PCT Publication No. WO 92/09690 (Garrard *et al.*); Fuchs *et al.*, Bio/Technology, 9: 1369-1372 (1991); Hay *et al.*, *Hum. Antibod. Hybridomas*, 3: 81-85 (1992); Huse *et al.*, *Science*, 246: 1275-1281 (1989); McCafferty *et al.*, *Nature*, 348: 552-554 (1990); Griffiths *et al.*, *EMBO J.*, 12: 725-734 (1993); Hawkins *et al.*, J. Mol. Biol., 226: 889-896 (1992); Clackson *et al.*, Nature, 352: 624-628 (1991); Gram *et al.*, *Proc. Natl. Acad. Sci. USA*, 89: 3576-3580 (1992); Garrard *et al.*, *Bio/Technology*, 9: 1373-1377 (1991); Hoogenboom *et al.*, Nucl. Acids Res., 19: 4133-4137 (1991); Barbas *et al.*, *Proc. Natl. Acad. Sci. USA*, 88: 7978-7982 (1991); U.S. Patent Application Publication No. 2003/0186374; and PCT Publication No. WO 97/29131, the contents of each of which are incorporated herein by reference.

[0220] The recombinant antibody library may be from a subject immunized with UCH-L1, or a portion of UCH-L1. Alternatively, the recombinant antibody library may be from a naive subject, *i.e.*, one who has not been immunized with UCH-L1, such as a human antibody library from a human subject who has not been immunized with human UCH-L1. Antibodies of the invention are selected by screening the recombinant antibody library with the peptide comprising human UCH-L1 to thereby select those antibodies that recognize UCH-L1. Methods for conducting such screening and selection are well known in the art, such as described in the references in the preceding paragraph. To select antibodies of the invention having particular binding affinities for UCH-L1, such as those that dissociate from human

UCH-L1 with a particular K_{off} rate constant, the art-known method of surface plasmon resonance can be used to select antibodies having the desired K_{off} rate constant. To select antibodies of the invention having a particular neutralizing activity for hUCH-L1, such as those with a particular IC_{50}, standard methods known in the art for assessing the inhibition of UCH-L1 activity may be used.

[0221] In one aspect, the invention pertains to an isolated antibody, or an antigen-binding portion thereof, that binds human UCH-L1. Preferably, the antibody is a neutralizing antibody. In various embodiments, the antibody is a recombinant antibody or a monoclonal antibody.

[0222] For example, antibodies can also be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the polynucleotide sequences encoding them. Such phage can be utilized to display antigen-binding domains expressed from a repertoire or combinatorial antibody library (*e.g.*, human or murine). Phage expressing an antigen binding domain that binds the antigen of interest can be selected or identified with antigen, *e.g.*, using labeled antigen or antigen bound or captured to a solid surface or bead. Phage used in these methods are typically filamentous phage including fd and M13 binding domains expressed from phage with Fab, Fv, or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein. Examples of phage display methods that can be used to make the antibodies include those disclosed in Brinkmann *et al.*, *J. Immunol. Methods*, 182: 41-50 (1995); Ames *et al.*, J. Immunol. Methods, 184:177-186 (1995); Kettleborough *et al.*, *Eur. J. Immunol.*, 24: 952-958 (1994); Persic *et al.*, Gene, 187: 9-18 (1997); Burton *et al.*, Advances in *Immunology*, 57: 191-280 (1994); PCT Publication No. WO 92/01047; PCT Publication Nos. WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and U.S. Patent Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743; and 5,969,108.

[0223] As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies including human antibodies or any other desired antigen binding fragment, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, *e.g.*, as described in detail below. For example, techniques to recombinantly produce Fab, Fab', and $F(ab')_2$ fragments can also be employed using methods known in the art such as those

disclosed in PCT publication No. WO 92/22324; Mullinax *et al.*, *BioTechniques*, 12(6): 864-869 (1992); Sawai *et al.*, *Am. J. Reprod. Immunol.*, 34: 26-34 (1995); and Better *et al.*, *Science*, 240: 1041-1043 (1988). Examples of techniques which can be used to produce single-chain Fvs and antibodies include those described in U.S. Patent Nos. 4,946,778 and 5,258,498; Huston *et al.*, Methods in Enzymology, 203: 46-88 (1991); Shu *et al.*, *Proc. Natl. Acad. Sci. USA*, 90: 7995-7999 (1993); and Skerra *et al.*, Science, 240: 1038-1041 (1988).

[0224] Alternative to screening of recombinant antibody libraries by phage display, other methodologies known in the art for screening large combinatorial libraries can be applied to the identification of antibodies of the invention. One type of alternative expression system is one in which the recombinant antibody library is expressed as RNA-protein fusions, as described in PCT Publication No. WO 98/31700 (Szostak and Roberts), and in Roberts and Szostak, *Proc. Natl. Acad. Sci. USA*, 94: 12297-12302 (1997). In this system, a covalent fusion is created between an mRNA and the peptide or protein that it encodes by in vitro translation of synthetic mRNAs that carry puromycin, a peptidyl acceptor antibiotic, at their 3' end. Thus, a specific mRNA can be enriched from a complex mixture of mRNAs (e.g., a combinatorial library) based on the properties of the encoded peptide or protein, e.g., antibody, or portion thereof, such as binding of the antibody, or portion thereof, to the dual specificity antigen. Nucleic acid sequences encoding antibodies, or portions thereof, recovered from screening of such libraries can be expressed by recombinant means as described above (e.g., in mammalian host cells) and, moreover, can be subjected to further affinity maturation by either additional rounds of screening of mRNA-peptide fusions in which mutations have been introduced into the originally selected sequence(s), or by other methods for affinity maturation in vitro of recombinant antibodies, as described above. A preferred example of this methodology is PROfusion display technology.

[0225] In another approach, the antibodies can also be generated using yeast display methods known in the art. In yeast display methods, genetic methods are used to tether antibody domains to the yeast cell wall and display them on the surface of yeast. In particular, such yeast can be utilized to display antigen-binding domains expressed from a repertoire or combinatorial antibody library (e.g., human or murine). Examples of yeast display methods that can be used to make the antibodies include those disclosed in U.S. Patent No. 6,699,658 (Wittrup *et al.*) incorporated herein by reference.

d. Production of Recombinant UCH-L1 Antibodies

[0226] Antibodies may be produced by any of a number of techniques known in the art. For example, expression from host cells, wherein expression vector(s) encoding the heavy and light chains is (are) transfected into a host cell by standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, e.g., electroporation, calcium-phosphate precipitation, DEAE-dextran transfection, and the like. Although it is possible to express the antibodies of the invention in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells is preferable, and most preferable in mammalian host cells, because such eukaryotic cells (and in particular mammalian cells) are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody.

[0227] Exemplary mammalian host cells for expressing the recombinant antibodies of the invention include Chinese Hamster Ovary (CHO cells) (including dhfr-CHO cells, described in Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77: 4216-4220 (1980), used with a DHFR selectable marker, *e.g.*, as described in Kaufman and Sharp, *J. Mol. Biol.*, 159: 601-621 (1982), NS0 myeloma cells, COS cells, and SP2 cells. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

[0228] Host cells can also be used to produce functional antibody fragments, such as Fab fragments or scFv molecules. It will be understood that variations on the above procedure may be performed. For example, it may be desirable to transfect a host cell with DNA encoding functional fragments of either the light chain and/or the heavy chain of an antibody of this invention. Recombinant DNA technology may also be used to remove some, or all, of the DNA encoding either or both of the light and heavy chains that is not necessary for binding to the antigens of interest. The molecules expressed from such truncated DNA molecules are also encompassed by the antibodies of the invention. In addition, bifunctional antibodies may be produced in which one heavy and one light chain are an antibody of the invention (*i.e.*, binds human UCH-L1) and the other heavy and light chain are specific for an

antigen other than human UCH-L1 by crosslinking an antibody of the invention to a second antibody by standard chemical crosslinking methods.

[0229] In a preferred system for recombinant expression of an antibody, or antigen-binding portion thereof, of the invention, a recombinant expression vector encoding both the antibody heavy chain and the antibody light chain is introduced into dhfr-CHO cells by calcium phosphate-mediated transfection. Within the recombinant expression vector, the antibody heavy and light chain genes are each operatively linked to CMV enhancer/AdMLP promoter regulatory elements to drive high levels of transcription of the genes. The recombinant expression vector also carries a DHFR gene, which allows for selection of CHO cells that have been transfected with the vector using methotrexate selection/amplification. The selected transformant host cells are cultured to allow for expression of the antibody heavy and light chains and intact antibody is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture the host cells, and recover the antibody from the culture medium. Still further, the invention provides a method of synthesizing a recombinant antibody of the invention by culturing a host cell of the invention in a suitable culture medium until a recombinant antibody of the invention is synthesized. The method can further comprise isolating the recombinant antibody from the culture medium.

(1) Humanized Antibody

[0230] The humanized antibody may be an antibody or a variant, derivative, analog or portion thereof which immunospecifically binds to an antigen of interest and which comprises a framework (FR) region having substantially the amino acid sequence of a human antibody and a complementary determining region (CDR) having substantially the amino acid sequence of a non-human antibody. The humanized antibody may be from a non-human species antibody that binds the desired antigen having one or more complementarity determining regions (CDRs) from the non-human species and framework regions from a human immunoglobulin molecule.

[0231] As used herein, the term "substantially" in the context of a CDR refers to a CDR having an amino acid sequence at least 90%, at least 95%, at least 98% or at least 99% identical to the amino acid sequence of a non-human antibody CDR. A humanized antibody comprises substantially all of at least one, and typically two, variable domains (Fab, Fab', $F(ab')_2$, FabC, Fv) in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin (*i.e.*, donor antibody) and all or substantially all of the framework regions are those of a human immunoglobulin consensus sequence. According to

one aspect, a humanized antibody also comprises at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. In some embodiments, a humanized antibody contains both the light chain as well as at least the variable domain of a heavy chain. The antibody also may include the CH1, hinge, CH2, CH3, and CH4 regions of the heavy chain. In some embodiments, a humanized antibody only contains a humanized light chain. In some embodiments, a humanized antibody only contains a humanized heavy chain. In specific embodiments, a humanized antibody only contains a humanized variable domain of a light chain and/or of a heavy chain.

[0232] The humanized antibody can be selected from any class of immunoglobulins, including IgM, IgG, IgD, IgA and IgE, and any isotype, including without limitation IgG 1, IgG2, IgG3, and IgG4. The humanized antibody may comprise sequences from more than one class or isotype, and particular constant domains may be selected to optimize desired effector functions using techniques well-known in the art.

[0233] The framework and CDR regions of a humanized antibody need not correspond precisely to the parental sequences, *e.g.*, the donor antibody CDR or the consensus framework may be mutagenized by substitution, insertion and/or deletion of at least one amino acid residue so that the CDR or framework residue at that site does not correspond to either the donor antibody or the consensus framework. In one embodiment, such mutations, however, will not be extensive. Usually, at least 90%, at least 95%, at least 98%, or at least 99% of the humanized antibody residues will correspond to those of the parental FR and CDR sequences. As used herein, the term "consensus framework" refers to the framework region in the consensus immunoglobulin sequence. As used herein, the term "consensus immunoglobulin sequence" refers to the sequence formed from the most frequently occurring amino acids (or nucleotides) in a family of related immunoglobulin sequences (See *e.g.*, Winnaker, *From Genes to Clones* (Verlagsgesellschaft, Weinheim, Germany 1987)). In a family of immunoglobulins, each position in the consensus sequence is occupied by the amino acid occurring most frequently at that position in the family. If two amino acids occur equally frequently, either can be included in the consensus sequence.

[0234] The humanized antibody may be designed to minimize unwanted immunological response toward rodent anti-human antibodies, which limits the duration and effectiveness of therapeutic applications of those moieties in human recipients. The humanized antibody may have one or more amino acid residues introduced into it from a source that is non-human. These non-human residues are often referred to as “import” residues, which are typically taken from a variable domain. Humanization may be performed by substituting

hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. For example, see U.S. Patent No. 4,816,567, the contents of which are herein incorporated by reference. The humanized antibody may be a human antibody in which some hypervariable region residues, and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies. Humanization or engineering of antibodies of the present invention can be performed using any known method, such as but not limited to those described in U.S. Patent Nos. 5,723,323; 5,976,862; 5,824,514; 5,817,483; 5,814,476; 5,763,192; 5,723,323; 5,766,886; 5,714,352; 6,204,023; 6,180,370; 5,693,762; 5,530,101; 5,585,089; 5,225,539; and 4,816,567.

[0235] The humanized antibody may retain high affinity for UCH-L1 and other favorable biological properties. The humanized antibody may be prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available. Computer programs are available that illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristics, such as increased affinity for UCH-L1, is achieved. In general, the hypervariable region residues may be directly and most substantially involved in influencing antigen binding.

[0236] As an alternative to humanization, human antibodies (also referred to herein as "fully human antibodies") can be generated. For example, it is possible to isolate human antibodies from libraries via PROfusion and/or yeast related technologies. It is also possible to produce transgenic animals (*e.g.*, mice that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. The humanized or fully human antibodies may be prepared according to

the methods described in U.S. Patent Nos. 5,770,429; 5,833,985; 5,837,243; 5,922,845; 6,017,517; 6,096,311; 6,111,166; 6,270,765; 6,303,755; 6,365,116; 6,410,690; 6,682,928; and 6,984,720, the contents each of which are herein incorporated by reference.

e. Anti-UCH-L1 antibodies

[0237] Anti-UCH-L1 antibodies may be generated using the techniques described above as well as using routine techniques known in the art. In some embodiments, the anti-UCH-L1 antibody may be an unconjugated UCH-L1 antibody, such as UCH-L1 antibodies available from United State Biological (Catalog Number: 031320), Cell Signaling Technology (Catalog Number: 3524), Sigma-Aldrich (Catalog Number: HPA005993), Santa Cruz Biotechnology, Inc. (Catalog Numbers: sc-58593 or sc-58594), R&D Systems (Catalog Number: MAB6007), Novus Biologicals (Catalog Number: NB600-1160), Biorbyt (Catalog Number: orb33715), Enzo Life Sciences, Inc. (Catalog Number: ADI-905-520-1), Bio-Rad (Catalog Number: VMA00004), BioVision (Catalog Number: 6130-50), Abcam (Catalog Numbers: ab75275 or ab104938), Invitrogen Antibodies (Catalog Numbers: 480012), ThermoFisher Scientific (Catalog Numbers: MA1-46079, MA5-17235, MA1-90008, or MA1-83428), EMD Millipore (Catalog Number: MABN48), or Sino Biological Inc. (Catalog Number: 50690-R011). The anti-UCH-L1 antibody may be conjugated to a fluorophore, such as conjugated UCH-L1 antibodies available from BioVision (Catalog Number: 6960-25) or Aviva Systems Biology (Cat. Nos. OAAF01904-FITC).

7. Methods for Measuring the Level of GFAP

[0238] In the methods described above, GFAP levels can be measured by any means, such as antibody dependent methods, such as immunoassays, protein immunoprecipitation, immunoelectrophoresis, chemical analysis, SDS-PAGE and Western blot analysis, or protein immunostaining, electrophoresis analysis, a protein assay, a competitive binding assay, a functional protein assay, or chromatography or spectrometry methods, such as high-performance liquid chromatography (HPLC) or liquid chromatography–mass spectrometry (LC/MS). Also, the assay can be employed in clinical chemistry format such as would be known by one skilled in the art.

[0239] In some embodiments, measuring the level of GFAP includes contacting the sample with a first specific binding member and second specific binding member. In some embodiments the first specific binding member is a capture antibody and the second specific binding member is a detection antibody. In some embodiments, measuring the level of GFAP

includes contacting the sample, either simultaneously or sequentially, in any order: (1) a capture antibody (e.g., GFAP-capture antibody), which binds to an epitope on GFAP or GFAP fragment to form a capture antibody-GFAP antigen complex (e.g., GFAP-capture antibody-GFAP antigen complex), and (2) a detection antibody (e.g., GFAP-detection antibody), which includes a detectable label and binds to an epitope on GFAP that is not bound by the capture antibody, to form a GFAP antigen-detection antibody complex (e.g., GFAP antigen-GFAP-detection antibody complex), such that a capture antibody-GFAP antigen-detection antibody complex (e.g., GFAP-capture antibody-GFAP antigen-GFAP-detection antibody complex) is formed, and measuring the amount or concentration of GFAP in the sample based on the signal generated by the detectable label in the capture antibody-GFAP antigen-detection antibody complex.

[0240] In some embodiments, the first specific binding member is immobilized on a solid support. In some embodiments, the second specific binding member is immobilized on a solid support. In some embodiments, the first specific binding member is a GFAP antibody as described below.

[0241] In some embodiments, the sample is diluted or undiluted. The sample can be from about 1 to about 25 microliters, about 1 to about 24 microliters, about 1 to about 23 microliters, about 1 to about 22 microliters, about 1 to about 21 microliters, about 1 to about 20 microliters, about 1 to about 18 microliters, about 1 to about 17 microliters, about 1 to about 16 microliters, about 15 microliters or about 1 microliter, about 2 microliters, about 3 microliters, about 4 microliters, about 5 microliters, about 6 microliters, about 7 microliters, about 8 microliters, about 9 microliters, about 10 microliters, about 11 microliters, about 12 microliters, about 13 microliters, about 14 microliters, about 15 microliters, about 16 microliters, about 17 microliters, about 18 microliters, about 19 microliters, about 20 microliters, about 21 microliters, about 22 microliters, about 23 microliters, about 24 microliters or about 25 microliters. In some embodiments, the sample is from about 1 to about 150 microliters or less or from about 1 to about 25 microliters or less.

[0242] Some instruments (such as, for example the Abbott Laboratories instrument ARCHITECT®, and other core laboratory instruments) other than a point-of-care device may be capable of measuring levels of GFAP in a sample higher or greater than 25,000 pg/mL.

[0243] Other methods of detection include the use of or can be adapted for use on a nanopore device or nanowell device. Examples of nanopore devices are described in International Patent Publication No. WO 2016/161402, which is hereby incorporated by

reference in its entirety. Examples of nanowell device are described in International Patent Publication No. WO 2016/161400, which is hereby incorporated by reference in its entirety

8. GFAP Antibodies

[0244] The methods described herein may use an isolated antibody that specifically binds to Glial fibrillary acidic protein ("GFAP") (or fragments thereof), referred to as "GFAP antibody." The GFAP antibodies can be used to assess the GFAP status as a measure of traumatic brain injury, detect the presence of GFAP in a sample, quantify the amount of GFAP present in a sample, or detect the presence of and quantify the amount of GFAP in a sample.

a. Glial fibrillary acidic protein (GFAP)

[0245] Glial fibrillary acidic protein (GFAP) is a 50 kDa intracytoplasmic filamentous protein that constitutes a portion of the cytoskeleton in astrocytes, and it has proved to be the most specific marker for cells of astrocytic origin. GFAP protein is encoded by the GFAP gene in humans. GFAP is the principal intermediate filament of mature astrocytes. In the central rod domain of the molecule, GFAP shares considerable structural homology with the other intermediate filaments. GFAP is involved in astrocyte motility and shape by providing structural stability to astrocytic processes. Glial fibrillary acidic protein and its breakdown products (GFAP-BDP) are brain-specific proteins released into the blood as part of the pathophysiological response after traumatic brain injury (TBI). Following injury to the human CNS caused by trauma, genetic disorders, or chemicals, astrocytes proliferate and show extensive hypertrophy of the cell body and processes, and GFAP is markedly upregulated. In contrast, with increasing astrocyte malignancy, there is a progressive loss of GFAP production. GFAP can also be detected in Schwann cells, enteric glia cells, salivary gland neoplasms, metastasizing renal carcinomas, epiglottic cartilage, pituicytes, immature oligodendrocytes, papillary meningiomas, and myoepithelial cells of the breast.

[0246] Human GFAP may have the following amino acid sequence:

[0247] MERRRITSAARRSYVSSGEMMVGGLAPGRRLGPGTRLSLARMPPPLPTRV DFSLAGALNAGFKETRASERAEMMELNDRFASYIEKVRFLEQQNKALAAELNQLRA KEPTKLADVYQAELRELRLRLDQLTANSARLEVERDNLAQDLATVRQKLQDETNLR LEAENNLAAYRQEADEATLARLDLERKIESLEEEIRFLRKIHEEEVRELQEQLARQQV HVELDVAKPDLTAALKEIRTQYEAMASSNMHEAEEWYRSKFADLTDAAARNAELL RQAKHEANDYRRQLQSLTCDLESLRGTNESLERQMREQEERHVREAASYQEALARL

EEEGQSLKDEMARHLQEYQDLLNVKLALDIEIATYRKLLEGEENRITIPVQTFSNLQIR ETSLDTKSVSEGHLKRNIVVKTVEMRDGEVIKESKQEHKDVM (SEQ ID NO: 2).

[0248] The human GFAP may be a fragment or variant of SEQ ID NO: 2. The fragment of GFAP may be between 5 and 400 amino acids, between 10 and 400 amino acids, between 50 and 400 amino acids, between 60 and 400 amino acids, between 65 and 400 amino acids, between 100 and 400 amino acids, between 150 and 400 amino acids, between 100 and 300 amino acids, or between 200 and 300 amino acids in length. The fragment may comprise a contiguous number of amino acids from SEQ ID NO: 2. The human GFAP fragment or variant of SEQ ID NO: 2 may be a GFAP breakdown product (BDP). The GFAP BDP may be 38 kDa, 42 kDa (fainter 41 kDa), 47 kDa (fainter 45 kDa); 25 kDa (fainter 23 kDa); 19 kDa, or 20 kDa.

b. GFAP-Recognizing Antibody

[0249] The antibody is an antibody that binds to GFAP, a fragment thereof, an epitope of GFAP, or a variant thereof. The antibody may be a fragment of the anti-GFAP antibody or a variant or a derivative thereof. The antibody may be a polyclonal or monoclonal antibody. The antibody may be a chimeric antibody, a single chain antibody, an affinity matured antibody, a human antibody, a humanized antibody, a fully human antibody or an antibody fragment, such as a Fab fragment, or a mixture thereof. Antibody fragments or derivatives may comprise $F(ab')_2$, Fv or scFv fragments. The antibody derivatives can be produced by peptidomimetics. Further, techniques described for the production of single chain antibodies can be adapted to produce single chain antibodies.

[0250] The anti-GFAP antibodies may be a chimeric anti-GFAP or humanized anti-GFAP antibody. In one embodiment, both the humanized antibody and chimeric antibody are monovalent. In one embodiment, both the humanized antibody and chimeric antibody comprise a single Fab region linked to an Fc region.

[0251] Human antibodies may be derived from phage-display technology or from transgenic mice that express human immunoglobulin genes. The human antibody may be generated as a result of a human in vivo immune response and isolated. See, for example, Funaro et al., BMC Biotechnology, 2008(8):85. Therefore, the antibody may be a product of the human and not animal repertoire. Because it is of human origin, the risks of reactivity against self-antigens may be minimized. Alternatively, standard yeast display libraries and display technologies may be used to select and isolate human anti-GFAP antibodies. For example, libraries of naïve human single chain variable fragments (scFv) may be used to

select human anti-GFAP antibodies. Transgenic animals may be used to express human antibodies.

[0252] Humanized antibodies may be antibody molecules from non-human species antibody that binds the desired antigen having one or more complementarity determining regions (CDRs) from the non-human species and framework regions from a human immunoglobulin molecule.

[0253] The antibody is distinguishable from known antibodies in that it possesses different biological function(s) than those known in the art.

(1) Epitope

[0254] The antibody may immunospecifically bind to GFAP (SEQ ID NO: 2), a fragment thereof, or a variant thereof. The antibody may immunospecifically recognize and bind at least three amino acids, at least four amino acids, at least five amino acids, at least six amino acids, at least seven amino acids, at least eight amino acids, at least nine amino acids, or at least ten amino acids within an epitope region. The antibody may immunospecifically recognize and bind to an epitope that has at least three contiguous amino acids, at least four contiguous amino acids, at least five contiguous amino acids, at least six contiguous amino acids, at least seven contiguous amino acids, at least eight contiguous amino acids, at least nine contiguous amino acids, or at least ten contiguous amino acids of an epitope region.

c. Antibody Preparation/Production

[0255] Antibodies may be prepared by any of a variety of techniques, including those well known to those skilled in the art. In general, antibodies can be produced by cell culture techniques, including the generation of monoclonal antibodies via conventional techniques, or via transfection of antibody genes, heavy chains, and/or light chains into suitable bacterial or mammalian cell hosts, in order to allow for the production of antibodies, wherein the antibodies may be recombinant. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, e.g., electroporation, calcium-phosphate precipitation, DEAE-dextran transfection and the like. Although it is possible to express the antibodies in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells is preferable, and most preferable in mammalian host cells, because such eukaryotic cells (and in particular mammalian cells) are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody.

[0256] Exemplary mammalian host cells for expressing the recombinant antibodies include Chinese Hamster Ovary (CHO cells) (including dhfr-CHO cells, described in Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77: 4216-4220 (1980)), used with a DHFR selectable marker, e.g., as described in Kaufman and Sharp, *J. Mol. Biol.*, 159: 601-621 (1982), NS0 myeloma cells, COS cells, and SP2 cells. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

[0257] Host cells can also be used to produce functional antibody fragments, such as Fab fragments or scFv molecules. It will be understood that variations on the above procedure may be performed. For example, it may be desirable to transfect a host cell with DNA encoding functional fragments of either the light chain and/or the heavy chain of an antibody. Recombinant DNA technology may also be used to remove some, or all, of the DNA encoding either or both of the light and heavy chains that is not necessary for binding to the antigens of interest. The molecules expressed from such truncated DNA molecules are also encompassed by the antibodies. In addition, bifunctional antibodies may be produced in which one heavy and one light chain are an antibody (i.e., binds human GFAP) and the other heavy and light chain are specific for an antigen other than human GFAP by crosslinking an antibody to a second antibody by standard chemical crosslinking methods.

[0258] In a preferred system for recombinant expression of an antibody, or antigen-binding portion thereof, a recombinant expression vector encoding both the antibody heavy chain and the antibody light chain is introduced into dhfr-CHO cells by calcium phosphate-mediated transfection. Within the recombinant expression vector, the antibody heavy and light chain genes are each operatively linked to CMV enhancer/AdMLP promoter regulatory elements to drive high levels of transcription of the genes. The recombinant expression vector also carries a DHFR gene, which allows for selection of CHO cells that have been transfected with the vector using methotrexate selection/amplification. The selected transformant host cells are cultured to allow for expression of the antibody heavy and light chains and intact antibody is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture the host cells, and recover the antibody from the culture medium. Still further, the method of synthesizing a recombinant antibody may be by

culturing a host cell in a suitable culture medium until a recombinant antibody is synthesized. The method can further comprise isolating the recombinant antibody from the culture medium.

[0259] Methods of preparing monoclonal antibodies involve the preparation of immortal cell lines capable of producing antibodies having the desired specificity. Such cell lines may be produced from spleen cells obtained from an immunized animal. The animal may be immunized with GFAP or a fragment and/or variant thereof. The peptide used to immunize the animal may comprise amino acids encoding human Fc, for example the fragment crystallizable region or tail region of human antibody. The spleen cells may then be immortalized by, for example, fusion with a myeloma cell fusion partner. A variety of fusion techniques may be employed. For example, the spleen cells and myeloma cells may be combined with a nonionic detergent for a few minutes and then plated at low density on a selective medium that supports that growth of hybrid cells, but not myeloma cells. One such technique uses hypoxanthine, aminopterin, thymidine (HAT) selection. Another technique includes eletrofusion. After a sufficient time, usually about 1 to 2 weeks, colonies of hybrids are observed. Single colonies are selected and their culture supernatants tested for binding activity against the polypeptide. Hybridomas having high reactivity and specificity may be used.

[0260] Monoclonal antibodies may be isolated from the supernatants of growing hybridoma colonies. In addition, various techniques may be employed to enhance the yield, such as injection of the hybridoma cell line into the peritoneal cavity of a suitable vertebrate host, such as a mouse. Monoclonal antibodies may then be harvested from the ascites fluid or the blood. Contaminants may be removed from the antibodies by conventional techniques, such as chromatography, gel filtration, precipitation, and extraction. Affinity chromatography is an example of a method that can be used in a process to purify the antibodies.

[0261] The proteolytic enzyme papain preferentially cleaves IgG molecules to yield several fragments, two of which (the F(ab) fragments) each comprise a covalent heterodimer that includes an intact antigen-binding site. The enzyme pepsin is able to cleave IgG molecules to provide several fragments, including the $F(ab')_2$ fragment, which comprises both antigen-binding sites.

[0262] The Fv fragment can be produced by preferential proteolytic cleavage of an IgM, and on rare occasions IgG or IgA immunoglobulin molecules. The Fv fragment may be derived using recombinant techniques. The Fv fragment includes a non-covalent VH::VL

heterodimer including an antigen-binding site that retains much of the antigen recognition and binding capabilities of the native antibody molecule.

[0263] The antibody, antibody fragment, or derivative may comprise a heavy chain and a light chain complementarity determining region ("CDR") set, respectively interposed between a heavy chain and a light chain framework ("FR") set which provide support to the CDRs and define the spatial relationship of the CDRs relative to each other. The CDR set may contain three hypervariable regions of a heavy or light chain V region.

[0264] Other suitable methods of producing or isolating antibodies of the requisite specificity can be used, including, but not limited to, methods that select recombinant antibody from a peptide or protein library (e.g., but not limited to, a bacteriophage, ribosome, oligonucleotide, RNA, cDNA, yeast or the like, display library); e.g., as available from various commercial vendors such as Cambridge Antibody Technologies (Cambridgeshire, UK), MorphoSys (Martinsreid/Planegg, Del.), Biovation (Aberdeen, Scotland, UK) BioInvent (Lund, Sweden), using methods known in the art. See U.S. Patent Nos. 4,704,692; 5,723,323; 5,763,192; 5,814,476; 5,817,483; 5,824,514; 5,976,862. Alternative methods rely upon immunization of transgenic animals (e.g., SCID mice, Nguyen et al. (1997) *Microbiol. Immunol.* 41:901-907; Sandhu et al. (1996) *Crit. Rev. Biotechnol.* 16:95-118; Eren et al. (1998) *Immunol.* 93:154-161) that are capable of producing a repertoire of human antibodies, as known in the art and/or as described herein. Such techniques, include, but are not limited to, ribosome display (Hanes et al. (1997) *Proc. Natl. Acad. Sci. USA*, 94:4937-4942; Hanes et al. (1998) *Proc. Natl. Acad. Sci. USA*, 95:14130-14135); single cell antibody producing technologies (e.g., selected lymphocyte antibody method ("SLAM") (U.S. Patent No. 5,627,052, Wen et al. (1987) J. Immunol. 17:887-892; Babcook et al. (1996) *Proc. Natl. Acad. Sci. USA* 93:7843-7848); gel microdroplet and flow cytometry (Powell et al. (1990) Biotechnol. 8:333-337; One Cell Systems, (Cambridge, Mass).; Gray et al. (1995) J. Imm. Meth. 182:155-163; Kenny et al. (1995) Bio/Technol. 13:787-790); B-cell selection (Steenbakkers et al. (1994) *Molec. Biol. Reports* 19:125-134 (1994)).

[0265] An affinity matured antibody may be produced by any one of a number of procedures that are known in the art. For example, see Marks et al., *BioTechnology*, 10: 779-783 (1992) describes affinity maturation by VH and VL domain shuffling. Random mutagenesis of CDR and/or framework residues is described by Barbas et al., *Proc. Nat. Acad. Sci. USA*, 91: 3809-3813 (1994); Schier et al., Gene, 169: 147-155 (1995); Yelton et al., *J. Immunol.*, 155: 1994-2004 (1995); Jackson et al., *J. Immunol.*, 154(7): 3310-3319 (1995); Hawkins et al, *J. Mol. Biol.*, 226: 889-896 (1992). Selective mutation at selective

mutagenesis positions and at contact or hypermutation positions with an activity enhancing amino acid residue is described in U.S. Patent No. 6,914,128 B1.

[0266] Antibody variants can also be prepared using delivering a polynucleotide encoding an antibody to a suitable host such as to provide transgenic animals or mammals, such as goats, cows, horses, sheep, and the like, that produce such antibodies in their milk. These methods are known in the art and are described for example in U.S. Patent Nos. 5,827,690; 5,849,992; 4,873,316; 5,849,992; 5,994,616; 5,565,362; and 5,304,489.

[0267] Antibody variants also can be prepared by delivering a polynucleotide to provide transgenic plants and cultured plant cells (e.g., but not limited to tobacco, maize, and duckweed) that produce such antibodies, specified portions or variants in the plant parts or in cells cultured therefrom. For example, Cramer et al. (1999) *Curr. Top. Microbiol. Immunol.* 240:95-118 and references cited therein, describe the production of transgenic tobacco leaves expressing large amounts of recombinant proteins, e.g., using an inducible promoter. Transgenic maize have been used to express mammalian proteins at commercial production levels, with biological activities equivalent to those produced in other recombinant systems or purified from natural sources. See, e.g., Hood et al., *Adv. Exp. Med. Biol.* (1999) 464:127-147 and references cited therein. Antibody variants have also been produced in large amounts from transgenic plant seeds including antibody fragments, such as single chain antibodies (scFv's), including tobacco seeds and potato tubers. See, e.g., Conrad et al. (1998) *Plant Mol. Biol.* 38:101-109 and reference cited therein. Thus, antibodies can also be produced using transgenic plants, according to known methods.

[0268] Antibody derivatives can be produced, for example, by adding exogenous sequences to modify immunogenicity or reduce, enhance or modify binding, affinity, on-rate, off-rate, avidity, specificity, half-life, or any other suitable characteristic. Generally, part or all of the non-human or human CDR sequences are maintained while the non-human sequences of the variable and constant regions are replaced with human or other amino acids.

[0269] Small antibody fragments may be diabodies having two antigen-binding sites, wherein fragments comprise a heavy chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain (VH VL). See for example, EP 404,097; WO 93/11161; and Hollinger et al., (1993) *Proc. Natl. Acad. Sci. USA* 90:6444-6448. By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. See also, U.S. Patent No. 6,632,926 to Chen et al. which is hereby incorporated by reference in its entirety and discloses antibody variants that have one or more

amino acids inserted into a hypervariable region of the parent antibody and a binding affinity for a target antigen which is at least about two fold stronger than the binding affinity of the parent antibody for the antigen.

[0270] The antibody may be a linear antibody. The procedure for making a linear antibody is known in the art and described in Zapata et al. (1995) *Protein Eng.* 8(10):1057-1062. Briefly, these antibodies comprise a pair of tandem Fd segments (VH-CH1-VH-CH1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

[0271] The antibodies may be recovered and purified from recombinant cell cultures by known methods including, but not limited to, protein A purification, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. High performance liquid chromatography ("HPLC") can also be used for purification.

[0272] It may be useful to detectably label the antibody. Methods for conjugating antibodies to these agents are known in the art. For the purpose of illustration only, antibodies can be labeled with a detectable moiety such as a radioactive atom, a chromophore, a fluorophore, or the like. Such labeled antibodies can be used for diagnostic techniques, either in vivo, or in an isolated test sample. They can be linked to a cytokine, to a ligand, to another antibody. Suitable agents for coupling to antibodies to achieve an anti-tumor effect include cytokines, such as interleukin 2 (IL-2) and Tumor Necrosis Factor (TNF); photosensitizers, for use in photodynamic therapy, including aluminum (III) phthalocyanine tetrasulfonate, hematoporphyrin, and phthalocyanine; radionuclides, such as iodine-131 (131I), yttrium-90 (90Y), bismuth-212 (212Bi), bismuth-213 (213Bi), technetium-99m (99mTc), rhenium-186 (186Re), and rhenium-188 (188Re); antibiotics, such as doxorubicin, adriamycin, daunorubicin, methotrexate, daunomycin, neocarzinostatin, and carboplatin; bacterial, plant, and other toxins, such as diphtheria toxin, pseudomonas exotoxin A, staphylococcal enterotoxin A, abrin-A toxin, ricin A (deglycosylated ricin A and native ricin A), TGF-alpha toxin, cytotoxin from chinese cobra (naja naja atra), and gelonin (a plant toxin); ribosome inactivating proteins from plants, bacteria and fungi, such as restrictocin (a ribosome inactivating protein produced by Aspergillus restrictus), saporin (a ribosome inactivating protein from Saponaria officinalis), and RNase; tyrosine kinase inhibitors; ly207702 (a difluorinated purine nucleoside); liposomes containing anti cystic

agents (e.g., antisense oligonucleotides, plasmids which encode for toxins, methotrexate, etc.); and other antibodies or antibody fragments, such as F(ab).

[0273] Antibody production via the use of hybridoma technology, the selected lymphocyte antibody method (SLAM), transgenic animals, and recombinant antibody libraries is described in more detail below.

(1) Anti-GFAP Monoclonal Antibodies Using Hybridoma Technology

[0274] Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow et al., Antibodies: *A Laboratory Manual*, second edition, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1988); Hammerling, et al., *In Monoclonal Antibodies and T-Cell Hybridomas*, (Elsevier, N.Y., 1981). It is also noted that the term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. The term "monoclonal antibody" refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced.

[0275] Methods of generating monoclonal antibodies as well as antibodies produced by the method may comprise culturing a hybridoma cell secreting an antibody of the invention wherein, preferably, the hybridoma is generated by fusing splenocytes isolated from an animal, e.g., a rat or a mouse, immunized with GFAP with myeloma cells and then screening the hybridomas resulting from the fusion for hybridoma clones that secrete an antibody able to bind a polypeptide of the invention. Briefly, rats can be immunized with a GFAP antigen. In a preferred embodiment, the GFAP antigen is administered with an adjuvant to stimulate the immune response. Such adjuvants include complete or incomplete Freund's adjuvant, RIBI (muramyl dipeptides) or ISCOM (immunostimulating complexes). Such adjuvants may protect the polypeptide from rapid dispersal by sequestering it in a local deposit, or they may contain substances that stimulate the host to secrete factors that are chemotactic for macrophages and other components of the immune system. Preferably, if a polypeptide is being administered, the immunization schedule will involve two or more administrations of the polypeptide, spread out over several weeks; however, a single administration of the polypeptide may also be used.

[0276] After immunization of an animal with a GFAP antigen, antibodies and/or antibody-producing cells may be obtained from the animal. An anti-GFAP antibody-containing serum is obtained from the animal by bleeding or sacrificing the animal. The serum may be used as

it is obtained from the animal, an immunoglobulin fraction may be obtained from the serum, or the anti-GFAP antibodies may be purified from the serum. Serum or immunoglobulins obtained in this manner are polyclonal, thus having a heterogeneous array of properties.

[0277] Once an immune response is detected, e.g., antibodies specific for the antigen GFAP are detected in the rat serum, the rat spleen is harvested and splenocytes isolated. The splenocytes are then fused by well-known techniques to any suitable myeloma cells, for example, cells from cell line SP20 available from the American Type Culture Collection (ATCC, Manassas, Va., US). Hybridomas are selected and cloned by limited dilution. The hybridoma clones are then assayed by methods known in the art for cells that secrete antibodies capable of binding GFAP. Ascites fluid, which generally contains high levels of antibodies, can be generated by immunizing rats with positive hybridoma clones.

[0278] In another embodiment, antibody-producing immortalized hybridomas may be prepared from the immunized animal. After immunization, the animal is sacrificed and the splenic B cells are fused to immortalized myeloma cells as is well known in the art. See, e.g., Harlow and Lane, supra. In a preferred embodiment, the myeloma cells do not secrete immunoglobulin polypeptides (a non-secretory cell line). After fusion and antibiotic selection, the hybridomas are screened using GFAP, or a portion thereof, or a cell expressing GFAP. In a preferred embodiment, the initial screening is performed using an enzyme-linked immunosorbent assay (ELISA) or a radioimmunoassay (RIA), preferably an ELISA. An example of ELISA screening is provided in PCT Publication No. WO 00/37504.

[0279] Anti-GFAP antibody-producing hybridomas are selected, cloned, and further screened for desirable characteristics, including robust hybridoma growth, high antibody production, and desirable antibody characteristics. Hybridomas may be cultured and expanded in vivo in syngeneic animals, in animals that lack an immune system, e.g., nude mice, or in cell culture in vitro. Methods of selecting, cloning and expanding hybridomas are well known to those of ordinary skill in the art.

[0280] In a preferred embodiment, hybridomas are rat hybridomas. In another embodiment, hybridomas are produced in a non-human, non-rat species such as mice, sheep, pigs, goats, cattle, or horses. In yet another preferred embodiment, the hybridomas are human hybridomas, in which a human non-secretory myeloma is fused with a human cell expressing an anti-GFAP antibody.

[0281] Antibody fragments that recognize specific epitopes may be generated by known techniques. For example, Fab and $F(ab')_2$ fragments of the invention may be produced by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to

produce two identical Fab fragments) or pepsin (to produce an $F(ab')_2$ fragment). A $F(ab')_2$ fragment of an IgG molecule retains the two antigen-binding sites of the larger ("parent") IgG molecule, including both light chains (containing the variable light chain and constant light chain regions), the CH1 domains of the heavy chains, and a disulfide-forming hinge region of the parent IgG molecule. Accordingly, an $F(ab')_2$ fragment is still capable of crosslinking antigen molecules like the parent IgG molecule.

(2) Anti-GFAP Monoclonal Antibodies Using SLAM

[0282] In another aspect of the invention, recombinant antibodies are generated from single, isolated lymphocytes using a procedure referred to in the art as the selected lymphocyte antibody method (SLAM), as described in U.S. Patent No. 5,627,052; PCT Publication No. WO 92/02551; and Babcook et al., *Proc. Natl. Acad. Sci. USA*, 93: 7843-7848 (1996). In this method, single cells secreting antibodies of interest, e.g., lymphocytes derived from any one of the immunized animals are screened using an antigen-specific hemolytic plaque assay, wherein the antigen GFAP, a subunit of GFAP, or a fragment thereof, is coupled to sheep red blood cells using a linker, such as biotin, and used to identify single cells that secrete antibodies with specificity for GFAP. Following identification of antibody-secreting cells of interest, heavy- and light-chain variable region cDNAs are rescued from the cells by reverse transcriptase-PCR (RT-PCR) and these variable regions can then be expressed, in the context of appropriate immunoglobulin constant regions (e.g., human constant regions), in mammalian host cells, such as COS or CHO cells. The host cells transfected with the amplified immunoglobulin sequences, derived from in vivo selected lymphocytes, can then undergo further analysis and selection in vitro, for example, by panning the transfected cells to isolate cells expressing antibodies to GFAP. The amplified immunoglobulin sequences further can be manipulated in vitro, such as by in vitro affinity maturation method. See, for example, PCT Publication No. WO 97/29131 and PCT Publication No. WO 00/56772.

(3) Anti-GFAP Monoclonal Antibodies Using Transgenic Animals

[0283] In another embodiment of the invention, antibodies are produced by immunizing a non-human animal comprising some, or all, of the human immunoglobulin locus with a GFAP antigen. In an embodiment, the non-human animal is a XENOMOUSE® transgenic mouse, an engineered mouse strain that comprises large fragments of the human immunoglobulin loci and is deficient in mouse antibody production. See, e.g., Green et al., *Nature Genetics*, 7: 13-21 (1994) and U.S. Patent Nos. 5,916,771; 5,939,598; 5,985,615; 5,998,209; 6,075,181; 6,091,001; 6,114,598; and 6,130,364. See also PCT Publication Nos.

WO 91/10741; WO 94/02602; WO 96/34096; WO 96/33735; WO 98/16654; WO 98/24893; WO 98/50433; WO 99/45031; WO 99/53049; WO 00/09560; and WO 00/37504. The XENOMOUSE® transgenic mouse produces an adult-like human repertoire of fully human antibodies, and generates antigen-specific human monoclonal antibodies. The XENOMOUSE® transgenic mouse contains approximately 80% of the human antibody repertoire through introduction of megabase sized, germline configuration YAC fragments of the human heavy chain loci and x light chain loci. See Mendez et al., *Nature Genetics*, 15: 146-156 (1997), Green and Jakobovits, *J. Exp. Med.*, 188: 483-495 (1998), the disclosures of which are hereby incorporated by reference.

(4) Anti-GFAP Monoclonal Antibodies Using Recombinant Antibody Libraries

[0284] In vitro methods also can be used to make the antibodies of the invention, wherein an antibody library is screened to identify an antibody having the desired GFAP -binding specificity. Methods for such screening of recombinant antibody libraries are well known in the art and include methods described in, for example, U.S. Patent No. 5,223,409 (Ladner et al.); PCT Publication No. WO 92/18619 (Kang et al.); PCT Publication No. WO 91/17271 (Dower et al.); PCT Publication No. WO 92/20791 (Winter et al.); PCT Publication No. WO 92/15679 (Markland et al.); PCT Publication No. WO 93/01288 (Breitling et al.); PCT Publication No. WO 92/01047 (McCafferty et al.); PCT Publication No. WO 92/09690 (Garrard et al.); Fuchs et al., Bio/Technology, 9: 1369-1372 (1991); Hay et al., *Hum. Antibod. Hybridomas*, 3: 81-85 (1992); Huse et al., *Science*, 246: 1275-1281 (1989); McCafferty et al., *Nature*, 348: 552-554 (1990); Griffiths et al., *EMBO J.*, 12: 725-734 (1993); Hawkins et al., J. Mol. Biol., 226: 889-896 (1992); Clackson et al., Nature, 352: 624-628 (1991); Gram et al., *Proc. Natl. Acad. Sci. USA*, 89: 3576-3580 (1992); Garrard et al., *Bio/Technology*, 9: 1373-1377 (1991); Hoogenboom et al., Nucl. Acids Res., 19: 4133-4137 (1991); Barbas et al., *Proc. Natl. Acad. Sci. USA*, 88: 7978-7982 (1991); U.S. Patent Application Publication No. 2003/0186374; and PCT Publication No. WO 97/29131, the contents of each of which are incorporated herein by reference.

[0285] The recombinant antibody library may be from a subject immunized with GFAP, or a portion of GFAP. Alternatively, the recombinant antibody library may be from a naive subject, i.e., one who has not been immunized with GFAP, such as a human antibody library from a human subject who has not been immunized with human GFAP. Antibodies of the invention are selected by screening the recombinant antibody library with the peptide comprising human GFAP to thereby select those antibodies that recognize GFAP. Methods

for conducting such screening and selection are well known in the art, such as described in the references in the preceding paragraph. To select antibodies of the invention having particular binding affinities for GFAP, such as those that dissociate from human GFAP with a particular K_{off} rate constant, the art-known method of surface plasmon resonance can be used to select antibodies having the desired K_{off} rate constant. To select antibodies of the invention having a particular neutralizing activity for hGFAP, such as those with a particular IC_{50}, standard methods known in the art for assessing the inhibition of GFAP activity may be used.

[0286] In one aspect, the invention pertains to an isolated antibody, or an antigen-binding portion thereof, that binds human GFAP. Preferably, the antibody is a neutralizing antibody. In various embodiments, the antibody is a recombinant antibody or a monoclonal antibody.

[0287] For example, antibodies can also be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the polynucleotide sequences encoding them. Such phage can be utilized to display antigen-binding domains expressed from a repertoire or combinatorial antibody library (e.g., human or murine). Phage expressing an antigen binding domain that binds the antigen of interest can be selected or identified with antigen, e.g., using labeled antigen or antigen bound or captured to a solid surface or bead. Phage used in these methods are typically filamentous phage including fd and M13 binding domains expressed from phage with Fab, Fv, or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein. Examples of phage display methods that can be used to make the antibodies include those disclosed in Brinkmann et al., *J. Immunol. Methods*, 182: 41-50 (1995); Ames et al., J. Immunol. Methods, 184:177-186 (1995); Kettleborough et al., *Eur. J. Immunol.*, 24: 952-958 (1994); Persic et al., Gene, 187: 9-18 (1997); Burton et al., Advances in *Immunology*, 57: 191-280 (1994); PCT Publication No. WO 92/01047; PCT Publication Nos. WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/11236; WO 95/15982; WO 95/20401; and U.S. Patent Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743; and 5,969,108.

[0288] As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies including human antibodies or any other desired antigen binding fragment, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, e.g., as described in detail below. For example, techniques to recombinantly produce Fab, Fab', and

$F(ab')_2$ fragments can also be employed using methods known in the art such as those disclosed in PCT publication No. WO 92/22324; Mullinax et al., *BioTechniques*, 12(6): 864-869 (1992); Sawai et al., *Am. J. Reprod. Immunol.*, 34: 26-34 (1995); and Better et al., *Science*, 240: 1041-1043 (1988). Examples of techniques which can be used to produce single-chain Fvs and antibodies include those described in U.S. Patent Nos. 4,946,778 and 5,258,498; Huston et al., Methods in Enzymology, 203: 46-88 (1991); Shu et al., *Proc. Natl. Acad. Sci. USA*, 90: 7995-7999 (1993); and Skerra et al., Science, 240: 1038-1041 (1988).

[0289] Alternative to screening of recombinant antibody libraries by phage display, other methodologies known in the art for screening large combinatorial libraries can be applied to the identification of antibodies of the invention. One type of alternative expression system is one in which the recombinant antibody library is expressed as RNA-protein fusions, as described in PCT Publication No. WO 98/31700 (Szostak and Roberts), and in Roberts and Szostak, Proc. Natl. Acad. Sci. USA, 94: 12297-12302 (1997). In this system, a covalent fusion is created between an mRNA and the peptide or protein that it encodes by in vitro translation of synthetic mRNAs that carry puromycin, a peptidyl acceptor antibiotic, at their 3' end. Thus, a specific mRNA can be enriched from a complex mixture of mRNAs (e.g., a combinatorial library) based on the properties of the encoded peptide or protein, e.g., antibody, or portion thereof, such as binding of the antibody, or portion thereof, to the dual specificity antigen. Nucleic acid sequences encoding antibodies, or portions thereof, recovered from screening of such libraries can be expressed by recombinant means as described above (e.g., in mammalian host cells) and, moreover, can be subjected to further affinity maturation by either additional rounds of screening of mRNA-peptide fusions in which mutations have been introduced into the originally selected sequence(s), or by other methods for affinity maturation in vitro of recombinant antibodies, as described above. A preferred example of this methodology is PROfusion display technology.

[0290] In another approach, the antibodies can also be generated using yeast display methods known in the art. In yeast display methods, genetic methods are used to tether antibody domains to the yeast cell wall and display them on the surface of yeast. In particular, such yeast can be utilized to display antigen-binding domains expressed from a repertoire or combinatorial antibody library (e.g., human or murine). Examples of yeast display methods that can be used to make the antibodies include those disclosed in U.S. Patent No. 6,699,658 (Wittrup et al.) incorporated herein by reference.

d. Production of Recombinant GFAP Antibodies

[0291] Antibodies may be produced by any of a number of techniques known in the art. For example, expression from host cells, wherein expression vector(s) encoding the heavy and light chains is (are) transfected into a host cell by standard techniques. The various forms of the term "transfection" are intended to encompass a wide variety of techniques commonly used for the introduction of exogenous DNA into a prokaryotic or eukaryotic host cell, e.g., electroporation, calcium-phosphate precipitation, DEAE-dextran transfection, and the like. Although it is possible to express the antibodies of the invention in either prokaryotic or eukaryotic host cells, expression of antibodies in eukaryotic cells is preferable, and most preferable in mammalian host cells, because such eukaryotic cells (and in particular mammalian cells) are more likely than prokaryotic cells to assemble and secrete a properly folded and immunologically active antibody.

[0292] Exemplary mammalian host cells for expressing the recombinant antibodies of the invention include Chinese Hamster Ovary (CHO cells) (including dhfr-CHO cells, described in Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77: 4216-4220 (1980), used with a DHFR selectable marker, e.g., as described in Kaufman and Sharp, *J. Mol. Biol.*, 159: 601-621 (1982), NS0 myeloma cells, COS cells, and SP2 cells. When recombinant expression vectors encoding antibody genes are introduced into mammalian host cells, the antibodies are produced by culturing the host cells for a period of time sufficient to allow for expression of the antibody in the host cells or, more preferably, secretion of the antibody into the culture medium in which the host cells are grown. Antibodies can be recovered from the culture medium using standard protein purification methods.

[0293] Host cells can also be used to produce functional antibody fragments, such as Fab fragments or scFv molecules. It will be understood that variations on the above procedure may be performed. For example, it may be desirable to transfect a host cell with DNA encoding functional fragments of either the light chain and/or the heavy chain of an antibody of this invention. Recombinant DNA technology may also be used to remove some, or all, of the DNA encoding either or both of the light and heavy chains that is not necessary for binding to the antigens of interest. The molecules expressed from such truncated DNA molecules are also encompassed by the antibodies of the invention. In addition, bifunctional antibodies may be produced in which one heavy and one light chain are an antibody of the invention (i.e., binds human GFAP) and the other heavy and light chain are specific for an

antigen other than human GFAP by crosslinking an antibody of the invention to a second antibody by standard chemical crosslinking methods.

[0294] In a preferred system for recombinant expression of an antibody, or antigen-binding portion thereof, of the invention, a recombinant expression vector encoding both the antibody heavy chain and the antibody light chain is introduced into dhfr-CHO cells by calcium phosphate-mediated transfection. Within the recombinant expression vector, the antibody heavy and light chain genes are each operatively linked to CMV enhancer/AdMLP promoter regulatory elements to drive high levels of transcription of the genes. The recombinant expression vector also carries a DHFR gene, which allows for selection of CHO cells that have been transfected with the vector using methotrexate selection/amplification. The selected transformant host cells are cultured to allow for expression of the antibody heavy and light chains and intact antibody is recovered from the culture medium. Standard molecular biology techniques are used to prepare the recombinant expression vector, transfect the host cells, select for transformants, culture the host cells, and recover the antibody from the culture medium. Still further, the invention provides a method of synthesizing a recombinant antibody of the invention by culturing a host cell of the invention in a suitable culture medium until a recombinant antibody of the invention is synthesized. The method can further comprise isolating the recombinant antibody from the culture medium.

(1) Humanized Antibody

[0295] The humanized antibody may be an antibody or a variant, derivative, analog or portion thereof which immunospecifically binds to an antigen of interest and which comprises a framework (FR) region having substantially the amino acid sequence of a human antibody and a complementary determining region (CDR) having substantially the amino acid sequence of a non-human antibody. The humanized antibody may be from a non-human species antibody that binds the desired antigen having one or more complementarity determining regions (CDRs) from the non-human species and framework regions from a human immunoglobulin molecule.

[0296] As used herein, the term "substantially" in the context of a CDR refers to a CDR having an amino acid sequence at least 90%, at least 95%, at least 98% or at least 99% identical to the amino acid sequence of a non-human antibody CDR. A humanized antibody comprises substantially all of at least one, and typically two, variable domains (Fab, Fab', $F(ab')_2$, FabC, Fv) in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin (i.e., donor antibody) and all or substantially all of the framework regions are those of a human immunoglobulin consensus sequence. According to

one aspect, a humanized antibody also comprises at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. In some embodiments, a humanized antibody contains both the light chain as well as at least the variable domain of a heavy chain. The antibody also may include the CH1, hinge, CH2, CH3, and CH4 regions of the heavy chain. In some embodiments, a humanized antibody only contains a humanized light chain. In some embodiments, a humanized antibody only contains a humanized heavy chain. In specific embodiments, a humanized antibody only contains a humanized variable domain of a light chain and/or of a heavy chain.

[0297] The humanized antibody can be selected from any class of immunoglobulins, including IgM, IgG, IgD, IgA and IgE, and any isotype, including without limitation IgG 1, IgG2, IgG3, and IgG4. The humanized antibody may comprise sequences from more than one class or isotype, and particular constant domains may be selected to optimize desired effector functions using techniques well-known in the art.

[0298] The framework and CDR regions of a humanized antibody need not correspond precisely to the parental sequences, e.g., the donor antibody CDR or the consensus framework may be mutagenized by substitution, insertion and/or deletion of at least one amino acid residue so that the CDR or framework residue at that site does not correspond to either the donor antibody or the consensus framework. In one embodiment, such mutations, however, will not be extensive. Usually, at least 90%, at least 95%, at least 98%, or at least 99% of the humanized antibody residues will correspond to those of the parental FR and CDR sequences. As used herein, the term "consensus framework" refers to the framework region in the consensus immunoglobulin sequence. As used herein, the term "consensus immunoglobulin sequence" refers to the sequence formed from the most frequently occurring amino acids (or nucleotides) in a family of related immunoglobulin sequences (See e.g., Winnaker, *From Genes to Clones* (Verlagsgesellschaft, Weinheim, Germany 1987)). In a family of immunoglobulins, each position in the consensus sequence is occupied by the amino acid occurring most frequently at that position in the family. If two amino acids occur equally frequently, either can be included in the consensus sequence.

[0299] The humanized antibody may be designed to minimize unwanted immunological response toward rodent anti-human antibodies, which limits the duration and effectiveness of therapeutic applications of those moieties in human recipients. The humanized antibody may have one or more amino acid residues introduced into it from a source that is non-human. These non-human residues are often referred to as “import” residues, which are typically taken from a variable domain. Humanization may be performed by substituting

hypervariable region sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. For example, see U.S. Patent No. 4,816,567, the contents of which are herein incorporated by reference. The humanized antibody may be a human antibody in which some hypervariable region residues, and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies. Humanization or engineering of antibodies of the present invention can be performed using any known method, such as but not limited to those described in U.S. Patent Nos. 5,723,323; 5,976,862; 5,824,514; 5,817,483; 5,814,476; 5,763,192; 5,723,323; 5,766,886; 5,714,352; 6,204,023; 6,180,370; 5,693,762; 5,530,101; 5,585,089; 5,225,539; and 4,816,567.

[0300] The humanized antibody may retain high affinity for GFAP and other favorable biological properties. The humanized antibody may be prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available. Computer programs are available that illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristics, such as increased affinity for GFAP, is achieved. In general, the hypervariable region residues may be directly and most substantially involved in influencing antigen binding.

[0301] As an alternative to humanization, human antibodies (also referred to herein as "fully human antibodies") can be generated. For example, it is possible to isolate human antibodies from libraries via PROfusion and/or yeast related technologies. It is also possible to produce transgenic animals (e.g. mice that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. The humanized or fully human antibodies may be prepared according to

the methods described in U.S. Patent Nos. 5,770,429; 5,833,985; 5,837,243; 5,922,845; 6,017,517; 6,096,311; 6,111,166; 6,270,765; 6,303,755; 6,365,116; 6,410,690; 6,682,928; and 6,984,720, the contents each of which are herein incorporated by reference.

e. Anti-GFAP antibodies

[0302] Anti-GFAP antibodies may be generated using the techniques described above as well as using routine techniques known in the art. In some embodiments, the anti-GFAP antibody may be an unconjugated GFAP antibody, such as GFAP antibodies available from Dako (Catalog Number: M0761), ThermoFisher Scientific (Catalog Numbers: MA5-12023, A-21282, 13-0300, MA1-19170, MA1-19395, MA5-15086, MA5-16367, MA1-35377, MA1-06701, or MA1-20035), AbCam (Catalog Numbers: ab10062, ab4648, ab68428, ab33922, ab207165, ab190288, ab115898, or ab21837), EMD Millipore (Catalog Numbers: FCMAB257P, MAB360, MAB3402, 04-1031, 04-1062, MAB5628), Santa Cruz (Catalog Numbers: sc-166481, sc-166458, sc-58766, sc-56395, sc-51908, sc-135921, sc-71143, sc-65343, or sc-33673), Sigma-Aldrich (Catalog Numbers: G3893 or G6171) or Sino Biological Inc. (Catalog Number: 100140-R012-50). The anti-GFAP antibody may be conjugated to a fluorophore, such as conjugated GFAP antibodies available from ThermoFisher Scientific (Catalog Numbers: A-21295 or A-21294), EMD Millipore (Catalog Numbers: MAB3402X, MAB3402B, MAB3402B, or MAB3402C3) or AbCam (Catalog Numbers: ab49874 or ab194325).

9. Variations on Methods

[0303] The disclosed methods of determining the presence or amount of analyte of interest (UCH-L1 and/or GFAP) present in a sample may be as described herein. The methods may also be adapted in view of other methods for analyzing analytes. Examples of well-known variations include, but are not limited to, immunoassay, such as sandwich immunoassay (*e.g.*, monoclonal-monoclonal sandwich immunoassays, monoclonal-polyclonal sandwich immunoassays, including enzyme detection (enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA), competitive inhibition immunoassay (*e.g.*, forward and reverse), enzyme multiplied immunoassay technique (EMIT), a competitive binding assay, bioluminescence resonance energy transfer (BRET), one-step antibody detection assay, homogeneous assay, heterogeneous assay, capture on the fly assay, etc.

a. Immunoassay

[0304] The analyte of interest, and/or peptides of fragments thereof (e.g., UCH-L1 and/or GFAP, and/or peptides or fragments thereof, i.e., UCH-L1 and/or GFAP fragments), may be analyzed using UCH-L1 and/or GFAP antibodies in an immunoassay. The presence or amount of analyte (e.g., UCH-L1 and/or GFAP) can be determined using antibodies and detecting specific binding to the analyte (e.g., UCH-L1 and/or GFAP). For example, the antibody, or antibody fragment thereof, may specifically bind to the analyte (e.g., UCH-L1 and/or GFAP). If desired, one or more of the antibodies can be used in combination with one or more commercially available monoclonal/polyclonal antibodies. Such antibodies are available from companies such as R&D Systems, Inc. (Minneapolis, MN) and Enzo Life Sciences International, Inc. (Plymouth Meeting, PA).

[0305] The presence or amount of analyte (e.g., UCH-L1 and/or GFAP) present in a body sample may be readily determined using an immunoassay, such as sandwich immunoassay (e.g., monoclonal-monoclonal sandwich immunoassays, monoclonal-polyclonal sandwich immunoassays, including radioisotope detection (radioimmunoassay (RIA)) and enzyme detection (enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA) (e.g., Quantikine ELISA assays, R&D Systems, Minneapolis, MN)). An example of a point-of-care device that can be used is i-STAT® (Abbott, Laboratories, Abbott Park, IL). Other methods that can be used include a chemiluminescent microparticle immunoassay, in particular one employing the ARCHITECT® automated analyzer (Abbott Laboratories, Abbott Park, IL), as an example. Other methods include, for example, mass spectrometry, and immunohistochemistry (e.g., with sections from tissue biopsies), using anti-analyte (*e.g.*, anti-UCH-L1 and/or anti-GFAP) antibodies (monoclonal, polyclonal, chimeric, humanized, human, etc.) or antibody fragments thereof against analyte (e.g., UCH-L1 and/or GFAP). Other methods of detection include those described in, for example, U.S. Patent Nos. 6,143,576; 6,113,855; 6,019,944; 5,985,579; 5,947,124; 5,939,272; 5,922,615; 5,885,527; 5,851,776; 5,824,799; 5,679,526; 5,525,524; and 5,480,792, each of which is hereby incorporated by reference in its entirety. Specific immunological binding of the antibody to the analyte (e.g., UCH-L1 and/or GFAP) can be detected via direct labels, such as fluorescent or luminescent tags, metals and radionuclides attached to the antibody or via indirect labels, such as alkaline phosphatase or horseradish peroxidase.

[0306] The use of immobilized antibodies or antibody fragments thereof may be incorporated into the immunoassay. The antibodies may be immobilized onto a variety of

supports, such as magnetic or chromatographic matrix particles, the surface of an assay plate (such as microtiter wells), pieces of a solid substrate material, and the like. An assay strip can be prepared by coating the antibody or plurality of antibodies in an array on a solid support. This strip can then be dipped into the test sample and processed quickly through washes and detection steps to generate a measurable signal, such as a colored spot.

[0307] A homogeneous format may be used. For example, after the test sample is obtained from a subject, a mixture is prepared. The mixture contains the test sample being assessed for analyte (e.g., UCH-L1 and/or GFAP), a first specific binding partner, and a second specific binding partner. The order in which the test sample, the first specific binding partner, and the second specific binding partner are added to form the mixture is not critical. The test sample is simultaneously contacted with the first specific binding partner and the second specific binding partner. In some embodiments, the first specific binding partner and any UCH-L1 and/or GFAP contained in the test sample may form a first specific binding partner-analyte (*e.g.,* UCH-L1 and/or GFAP)-antigen complex and the second specific binding partner may form a first specific binding partner-analyte of interest (e.g., UCH-L1 and/or GFAP)-second specific binding partner complex. In some embodiments, the second specific binding partner and any UCH-L1 and/or GFAP contained in the test sample may form a second specific binding partner-analyte (e.g., UCH-L1)-antigen complex and the first specific binding partner may form a first specific binding partner-analyte of interest (e.g., UCH-L1 and/or GFAP)-second specific binding partner complex. The first specific binding partner may be an anti-analyte antibody (e.g., anti-UCH-L1 antibody that binds to an epitope having an amino acid sequence comprising at least three contiguous (3) amino acids of SEQ ID NO: 1 or anti-GFAP antibody that binds to an epitope having an amino acid sequence comprising at least three contiguous (3) amino acids of SEQ ID NO: 2). The second specific binding partner may be an anti-analyte antibody (e.g., anti-UCH-L1 antibody that binds to an epitope having an amino acid sequence comprising at least three contiguous (3) amino acids of SEQ ID NO: 1 or anti-GFAP antibody that binds to an epitope having an amino acid sequence comprising at least three contiguous (3) amino acids of SEQ ID NO: 2). Moreover, the second specific binding partner is labeled with or contains a detectable label as described above.

[0308] A heterogeneous format may be used. For example, after the test sample is obtained from a subject, a first mixture is prepared. The mixture contains the test sample being assessed for analyte (e.g., UCH-L1 and/or GFAP) and a first specific binding partner, wherein the first specific binding partner and any UCH-L1 and/or GFAP contained in the test

sample form a first specific binding partner-analyte (e.g., UCH-L1 and/or GFAP)-antigen complex. The first specific binding partner may be an anti-analyte antibody (e.g., anti-UCH-L1 antibody that binds to an epitope having an amino acid sequence comprising at least three contiguous (3) amino acids of SEQ ID NO: 1 or anti-GFAP antibody that binds to an epitope having an amino acid sequence comprising at least three contiguous (3) amino acids of SEQ ID NO: 2). The order in which the test sample and the first specific binding partner are added to form the mixture is not critical.

[0309] The first specific binding partner may be immobilized on a solid phase. The solid phase used in the immunoassay (for the first specific binding partner and, optionally, the second specific binding partner) can be any solid phase known in the art, such as, but not limited to, a magnetic particle, a bead, a test tube, a microtiter plate, a cuvette, a membrane, a scaffolding molecule, a film, a filter paper, a disc, and a chip. In those embodiments where the solid phase is a bead, the bead may be a magnetic bead or a magnetic particle. Magnetic beads/particles may be ferromagnetic, ferrimagnetic, paramagnetic, superparamagnetic or ferrofluidic. Exemplary ferromagnetic materials include Fe, Co, Ni, Gd, Dy, CrO_2, MnAs, MnBi, EuO, and NiO/Fe. Examples of ferrimagnetic materials include $NiFe_2O_4$, $CoFe_2O_4$, Fe_3O_4 (or $FeO{\cdot}Fe_2O_3$). Beads can have a solid core portion that is magnetic and is surrounded by one or more non-magnetic layers. Alternately, the magnetic portion can be a layer around a non-magnetic core. The solid support on which the first specific binding member is immobilized may be stored in dry form or in a liquid. The magnetic beads may be subjected to a magnetic field prior to or after contacting with the sample with a magnetic bead on which the first specific binding member is immobilized.

[0310] After the mixture containing the first specific binding partner-analyte (e.g., UCH-L1 or GFAP) antigen complex is formed, any unbound analyte (e.g., UCH-L1 and/or GFAP) is removed from the complex using any technique known in the art. For example, the unbound analyte (e.g., UCH-L1 and/or GFAP) can be removed by washing. Desirably, however, the first specific binding partner is present in excess of any analyte (e.g., UCH-L1 and/or GFAP) present in the test sample, such that all analyte (e.g., UCH-L1 and/or GFAP) that is present in the test sample is bound by the first specific binding partner.

[0311] After any unbound analyte (e.g., UCH-L1 and/or GFAP) is removed, a second specific binding partner is added to the mixture to form a first specific binding partner-analyte of interest (e.g., UCH-L1 and/or GFAP)-second specific binding partner complex. The second specific binding partner may be an anti-analyte antibody (e.g., anti-UCH-L1 antibody that binds to an epitope having an amino acid sequence comprising at least three

contiguous (3) amino acids of SEQ ID NO: 1 or anti-GFAP antibody that binds to an epitope having an amino acid sequence comprising at least three contiguous (3) amino acids of SEQ ID NO: 2). Moreover, the second specific binding partner is labeled with or contains a detectable label as described above.

[0312] The use of immobilized antibodies or antibody fragments thereof may be incorporated into the immunoassay. The antibodies may be immobilized onto a variety of supports, such as magnetic or chromatographic matrix particles (such as a magnetic bead), latex particles or modified surface latex particles, polymer or polymer film, plastic or plastic film, planar substrate, the surface of an assay plate (such as microtiter wells), pieces of a solid substrate material, and the like. An assay strip can be prepared by coating the antibody or plurality of antibodies in an array on a solid support. This strip can then be dipped into the test sample and processed quickly through washes and detection steps to generate a measurable signal, such as a colored spot.

(1) Sandwich immunoassay

[0313] A sandwich immunoassay measures the amount of antigen between two layers of antibodies (i.e., at least one capture antibody) and a detection antibody (i.e., at least one detection antibody). The capture antibody and the detection antibody bind to different epitopes on the antigen, e.g., analyte of interest such as UCH-L1 and/or GFAP. Desirably, binding of the capture antibody to an epitope does not interfere with binding of the detection antibody to an epitope. Either monoclonal or polyclonal antibodies may be used as the capture and detection antibodies in the sandwich immunoassay.

[0314] Generally, at least two antibodies are employed to separate and quantify analyte (e.g., UCH-L1 and/or GFAP) in a test sample. More specifically, the at least two antibodies bind to certain epitopes of analyte (e.g., UCH-L1 and/or GFAP) forming an immune complex which is referred to as a "sandwich". One or more antibodies can be used to capture the analyte (e.g., UCH-L1 and/or GFAP) in the test sample (these antibodies are frequently referred to as a "capture" antibody or "capture" antibodies) and one or more antibodies is used to bind a detectable (namely, quantifiable) label to the sandwich (these antibodies are frequently referred to as the "detection" antibody or "detection" antibodies). In a sandwich assay, the binding of an antibody to its epitope desirably is not diminished by the binding of any other antibody in the assay to its respective epitope. Antibodies are selected so that the one or more first antibodies brought into contact with a test sample suspected of containing analyte (e.g., UCH-L1 and/or GFAP) do not bind to all or part of an epitope recognized by

the second or subsequent antibodies, thereby interfering with the ability of the one or more second detection antibodies to bind to the analyte (*e.g.*, UCH-L1 and/or GFAP).

[0315] The antibodies may be used as a first antibody in said immunoassay. The antibody immunospecifically binds to epitopes on analyte (e.g., UCH-L1 and/or GFAP). In addition to the antibodies of the present invention, said immunoassay may comprise a second antibody that immunospecifically binds to epitopes that are not recognized or bound by the first antibody.

[0316] A test sample suspected of containing analyte (e.g., UCH-L1 and/or GFAP) can be contacted with at least one first capture antibody (or antibodies) and at least one second detection antibodies either simultaneously or sequentially. In the sandwich assay format, a test sample suspected of containing analyte (e.g., UCH-L1 and/or GFAP) is first brought into contact with the at least one first capture antibody that specifically binds to a particular epitope under conditions which allow the formation of a first antibody-analyte (e.g., UCH-L1 and/or GFAP) antigen complex. If more than one capture antibody is used, a first multiple capture antibody-UCH-L1 and/or GFAP antigen complex is formed. In a sandwich assay, the antibodies, preferably, the at least one capture antibody, are used in molar excess amounts of the maximum amount of analyte (e.g., UCH-L1 and/or GFAP) expected in the test sample. For example, from about 5 µg/mL to about 1 mg/mL of antibody per ml of microparticle coating buffer may be used.

i. Anti-UCH-L1 Capture Antibody

[0317] Optionally, prior to contacting the test sample with the at least one first capture antibody, the at least one first capture antibody can be bound to a solid support which facilitates the separation the first antibody-analyte (e.g., UCH-L1 and/or GFAP) complex from the test sample. Any solid support known in the art can be used, including but not limited to, solid supports made out of polymeric materials in the forms of wells, tubes, or beads (such as a microparticle). The antibody (or antibodies) can be bound to the solid support by adsorption, by covalent bonding using a chemical coupling agent or by other means known in the art, provided that such binding does not interfere with the ability of the antibody to bind analyte (e.g., UCH-L1 and/or GFAP). Moreover, if necessary, the solid support can be derivatized to allow reactivity with various functional groups on the antibody. Such derivatization requires the use of certain coupling agents such as, but not limited to, maleic anhydride, N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

[0318] After the test sample suspected of containing analyte (e.g., UCH-L1 and/or GFAP) is incubated in order to allow for the formation of a first capture antibody (or multiple antibody)-analyte (*e.g.*, UCH-L1 and/or GFAP) complex. The incubation can be carried out at a pH of from about 4.5 to about 10.0, at a temperature of from about 2°C to about 45°C, and for a period from at least about one (1) minute to about eighteen (18) hours, from about 2-6 minutes, from about 7 -12 minutes, from about 5-15 minutes, or from about 3-4 minutes.

ii. Detection Antibody

[0319] After formation of the first/multiple capture antibody-analyte (e.g., UCH-L1 and/or GFAP) complex, the complex is then contacted with at least one second detection antibody (under conditions that allow for the formation of a first/multiple antibody-analyte (e.g., UCH-L1 and/or GFAP) antigen-second antibody complex). In some embodiments, the test sample is contacted with the detection antibody simultaneously with the capture antibody. If the first antibody-analyte (e.g., UCH-L1 and/or GFAP) complex is contacted with more than one detection antibody, then a first/multiple capture antibody-analyte (e.g., UCH-L1 and/or GFAP)-multiple antibody detection complex is formed. As with first antibody, when the at least second (and subsequent) antibody is brought into contact with the first antibody-analyte (e.g., UCH-L1 and/or GFAP) complex, a period of incubation under conditions similar to those described above is required for the formation of the first/multiple antibody-analyte (e.g., UCH-L1 and/or GFAP)-second/multiple antibody complex. Preferably, at least one second antibody contains a detectable label. The detectable label can be bound to the at least one second antibody prior to, simultaneously with or after the formation of the first/multiple antibody-analyte (e.g., UCH-L1 and/or GFAP)-second/multiple antibody complex. Any detectable label known in the art can be used.

[0320] Chemiluminescent assays can be performed in accordance with the methods described in Adamczyk *et al.*, *Anal. Chim. Acta* 579(1): 61-67 (2006). While any suitable assay format can be used, a microplate chemiluminometer (Mithras LB-940, Berthold Technologies U.S.A., LLC, Oak Ridge, TN) enables the assay of multiple samples of small volumes rapidly. The chemiluminometer can be equipped with multiple reagent injectors using 96-well black polystyrene microplates (Costar #3792). Each sample can be added into a separate well, followed by the simultaneous/sequential addition of other reagents as determined by the type of assay employed. Desirably, the formation of pseudobases in neutral or basic solutions employing an acridinium aryl ester is avoided, such as by acidification. The chemiluminescent response is then recorded well-by-well. In this regard,

the time for recording the chemiluminescent response will depend, in part, on the delay between the addition of the reagents and the particular acridinium employed.

[0321] The order in which the test sample and the specific binding partner(s) are added to form the mixture for chemiluminescent assay is not critical. If the first specific binding partner is detectably labeled with an acridinium compound, detectably labeled first specific binding partner-antigen (e.g., UCH-L1 and/or GFAP) complexes form. Alternatively, if a second specific binding partner is used and the second specific binding partner is detectably labeled with an acridinium compound, detectably labeled first specific binding partner-analyte (e.g., UCH-L1 and/or GFAP)-second specific binding partner complexes form. Any unbound specific binding partner, whether labeled or unlabeled, can be removed from the mixture using any technique known in the art, such as washing.

[0322] Hydrogen peroxide can be generated in situ in the mixture or provided or supplied to the mixture before, simultaneously with, or after the addition of an above-described acridinium compound. Hydrogen peroxide can be generated in situ in a number of ways such as would be apparent to one skilled in the art.

[0323] Alternatively, a source of hydrogen peroxide can be simply added to the mixture. For example, the source of the hydrogen peroxide can be one or more buffers or other solutions that are known to contain hydrogen peroxide. In this regard, a solution of hydrogen peroxide can simply be added.

[0324] Upon the simultaneous or subsequent addition of at least one basic solution to the sample, a detectable signal, namely, a chemiluminescent signal, indicative of the presence of analyte (*e.g.*, UCH-L1 and/or GFAP) is generated. The basic solution contains at least one base and has a pH greater than or equal to 10, preferably, greater than or equal to 12. Examples of basic solutions include, but are not limited to, sodium hydroxide, potassium hydroxide, calcium hydroxide, ammonium hydroxide, magnesium hydroxide, sodium carbonate, sodium bicarbonate, calcium hydroxide, calcium carbonate, and calcium bicarbonate. The amount of basic solution added to the sample depends on the concentration of the basic solution. Based on the concentration of the basic solution used, one skilled in the art can easily determine the amount of basic solution to add to the sample. Other labels other than chemiluminescent labels can be employed. For instance, enzymatic labels (including but not limited to alkaline phosphatase) can be employed.

[0325] The chemiluminescent signal, or other signal, that is generated can be detected using routine techniques known to those skilled in the art. Based on the intensity of the signal generated, the amount of analyte of interest (e.g., UCH-L1 and/or GFAP) in the sample

can be quantified. Specifically, the amount of analyte (e.g., UCH-L1 and/or GFAP) in the sample is proportional to the intensity of the signal generated. The amount of analyte (e.g., UCH-L1 and/or GFAP) present can be quantified by comparing the amount of light generated to a standard curve for analyte (e.g., UCH-L1 and/or GFAP) or by comparison to a reference standard. The standard curve can be generated using serial dilutions or solutions of known concentrations of analyte (e.g., UCH-L1 and/or GFAP) by mass spectroscopy, gravimetric methods, and other techniques known in the art.

(2) Forward Competitive Inhibition Assay

[0326] In a forward competitive format, an aliquot of labeled analyte of interest (e.g., analyte (*e.g.*, UCH-L1 and/or GFAP) having a fluorescent label, a tag attached with a cleavable linker, etc.) of a known concentration is used to compete with analyte of interest (e.g., UCH-L1 and/or GFAP) in a test sample for binding to analyte of interest antibody (e.g., UCH-L1 and/or GFAP antibody).

[0327] In a forward competition assay, an immobilized specific binding partner (such as an antibody) can either be sequentially or simultaneously contacted with the test sample and a labeled analyte of interest, analyte of interest fragment or analyte of interest variant thereof. The analyte of interest peptide, analyte of interest fragment or analyte of interest variant can be labeled with any detectable label, including a detectable label comprised of tag attached with a cleavable linker. In this assay, the antibody can be immobilized on to a solid support. Alternatively, the antibody can be coupled to an antibody, such as an antispecies antibody, that has been immobilized on a solid support, such as a microparticle or planar substrate.

[0328] The labeled analyte of interest, the test sample and the antibody are incubated under conditions similar to those described above in connection with the sandwich assay format. Two different species of antibody-analyte of interest complexes may then be generated. Specifically, one of the antibody-analyte of interest complexes generated contains a detectable label (e.g., a fluorescent label, etc.) while the other antibody-analyte of interest complex does not contain a detectable label. The antibody-analyte of interest complex can be, but does not have to be, separated from the remainder of the test sample prior to quantification of the detectable label. Regardless of whether the antibody-analyte of interest complex is separated from the remainder of the test sample, the amount of detectable label in the antibody-analyte of interest complex is then quantified. The concentration of analyte of interest (such as membrane-associated analyte of interest, soluble analyte of interest, fragments of soluble analyte of interest, variants of analyte of interest (membrane-associated

or soluble analyte of interest) or any combinations thereof) in the test sample can then be determined, e.g., as described above.

(3) Reverse Competitive Inhibition Assay

[0329] In a reverse competition assay, an immobilized analyte of interest (e.g., UCH-L1 and/or GFAP) can either be sequentially or simultaneously contacted with a test sample and at least one labeled antibody.

[0330] The analyte of interest can be bound to a solid support, such as the solid supports discussed above in connection with the sandwich assay format.

[0331] The immobilized analyte of interest, test sample and at least one labeled antibody are incubated under conditions similar to those described above in connection with the sandwich assay format. Two different species analyte of interest-antibody complexes are then generated. Specifically, one of the analyte of interest-antibody complexes generated is immobilized and contains a detectable label (e.g., a fluorescent label, etc.) while the other analyte of interest-antibody complex is not immobilized and contains a detectable label. The non-immobilized analyte of interest-antibody complex and the remainder of the test sample are removed from the presence of the immobilized analyte of interest-antibody complex through techniques known in the art, such as washing. Once the non-immobilized analyte of interest antibody complex is removed, the amount of detectable label in the immobilized analyte of interest-antibody complex is then quantified following cleavage of the tag. The concentration of analyte of interest in the test sample can then be determined by comparing the quantity of detectable label as described above.

(4) One-Step Immunoassay or "Capture on the Fly" Assay

[0332] In a capture on the fly immunoassay, a solid substrate is pre-coated with an immobilization agent. The capture agent, the analyte (e.g., UCH-L1 and/or GFAP) and the detection agent are added to the solid substrate together, followed by a wash step prior to detection. The capture agent can bind the analyte (e.g., UCH-L1 and/or GFAP) and comprises a ligand for an immobilization agent. The capture agent and the detection agents may be antibodies or any other moiety capable of capture or detection as described herein or known in the art. The ligand may comprise a peptide tag and an immobilization agent may comprise an anti-peptide tag antibody. Alternately, the ligand and the immobilization agent may be any pair of agents capable of binding together so as to be employed for a capture on the fly assay (*e.g.*, specific binding pair, and others such as are known in the art). More than one analyte may be measured. In some embodiments, the solid substrate may be coated with an antigen and the analyte to be analyzed is an antibody.

[0333] In certain other embodiments, in a one-step immunoassay or "capture on the fly", a solid support (such as a microparticle) pre-coated with an immobilization agent (such as biotin, streptavidin, etc.) and at least a first specific binding member and a second specific binding member (which function as capture and detection reagents, respectively) are used. The first specific binding member comprises a ligand for the immobilization agent (for example, if the immobilization agent on the solid support is streptavidin, the ligand on the first specific binding member may be biotin) and also binds to the analyte of interest (e.g., UCH-L1 and/or GFAP). The second specific binding member comprises a detectable label and binds to an analyte of interest (e.g., UCH-L1 and/or GFAP). The solid support and the first and second specific binding members may be added to a test sample (either sequentially or simultaneously). The ligand on the first specific binding member binds to the immobilization agent on the solid support to form a solid support/first specific binding member complex. Any analyte of interest present in the sample binds to the solid support/first specific binding member complex to form a solid support/first specific binding member/analyte complex. The second specific binding member binds to the solid support/first specific binding member/analyte complex and the detectable label is detected. An optional wash step may be employed before the detection. In certain embodiments, in a one-step assay more than one analyte may be measured. In certain other embodiments, more than two specific binding members can be employed. In certain other embodiments, multiple detectable labels can be added. In certain other embodiments, multiple analytes of interest can be detected, or their amounts, levels or concentrations, measured, determined or assessed.

[0334] The use of a capture on the fly assay can be done in a variety of formats as described herein, and known in the art. For example the format can be a sandwich assay such as described above, but alternately can be a competition assay, can employ a single specific binding member, or use other variations such as are known.

10. Other Factors

[0335] The methods of diagnosing, prognosticating, and/or assessing, as described above, can further include using other factors for the diagnosis, prognostication, and assessment. In some embodiments, traumatic brain injury may be diagnosed using the Glasgow Coma Scale or the Extended Glasgow Outcome Scale (GOSE). Other tests, scales or indices can also be used either alone or in combination with the Glasgow Coma Scale. An example is the Ranchos Los Amigos Scale. The Ranchos Los Amigos Scale measures the levels of awareness, cognition, behavior and interaction with the environment. The Ranchos Los

Amigos Scale includes: Level I: No Response; Level II: Generalized Response; Level III: Localized Response; Level IV: Confused-agitated; Level V: Confused-inappropriate; Level VI: Confused-appropriate; Level VII: Automatic-appropriate; and Level VIII: Purposeful-appropriate.

11. Samples

[0336] In some embodiments, the sample is obtained after the human subject sustained an injury to the head caused by physical shaking, blunt impact by an external mechanical or other force that results in a closed or open head trauma, one or more falls, explosions or blasts or other types of blunt force trauma. In some embodiments, the sample is obtained after the human subject has ingested or been exposed to a chemical, toxin or combination of a chemical and toxin. Examples of such chemicals and/or toxins include, fires, molds, asbestos, pesticides and insecticides, organic solvents, paints, glues, gases (such as carbon monoxide, hydrogen sulfide, and cyanide), organic metals (such as methyl mercury, tetraethyl lead and organic tin) and/or one or more drugs of abuse. In some embodiments, the sample is obtained from a human subject that suffers from an autoimmune disease, a metabolic disorder, a brain tumor, hypoxia, one or more viruses, meningitis, hydrocephalus or combinations thereof.

[0337] In yet another embodiment, the methods described herein use samples that also can be used to determine whether or not a subject has or is at risk of developing mild traumatic brain injury by determining the levels of UCH-L1 and/or GFAP in a subject using the anti-UCH-L1 and/or anti-GFAP antibodies described below, or antibody fragments thereof. Thus, in particular embodiments, the disclosure also provides a method for determining whether a subject having, or at risk for, traumatic brain injuries, discussed herein and known in the art, is a candidate for therapy or treatment. Generally, the subject is at least one who: (i) has experienced an injury to the head; (ii) ingested and/or been exposed to one or more chemicals and/or toxins; (iii) suffers from an autoimmune disease, a metabolic disorder, a brain tumor, hypoxia, one or more viruses, meningitis, hydrocephalus or suffers from any combinations thereof; or (iv) any combinations of (i)-(iii); or, who has actually been diagnosed as having, or being at risk for TBI (such as, for example, subjects suffering from an autoimmune disease, a metabolic disorder, a brain tumor, hypoxia, one or more viruses, meningitis, hydrocephalus or combinations thereof), and/or who demonstrates an unfavorable (*i.e.*, clinically undesirable) concentration or amount of UCH-L1 and/or GFAP or UCH-L1 and/or GFAP fragment, as described herein.

b. Test or Biological Sample

[0338] As used herein, "sample", "test sample", "biological sample" refer to fluid sample containing or suspected of containing UCH-L1 and/or GFAP. The sample may be derived from any suitable source. In some cases, the sample may comprise a liquid, fluent particulate solid, or fluid suspension of solid particles. In some cases, the sample may be processed prior to the analysis described herein. For example, the sample may be separated or purified from its source prior to analysis; however, in certain embodiments, an unprocessed sample containing UCH-L1 and/or GFAP may be assayed directly. In a particular example, the source of UCH-L1 and/or GFAP is a human bodily substance (e.g., bodily fluid, blood such as whole blood, serum, plasma, urine, saliva, sweat, sputum, semen, mucus, lacrimal fluid, lymph fluid, amniotic fluid, interstitial fluid, lung lavage, cerebrospinal fluid, feces, tissue, organ, or the like). Tissues may include, but are not limited to skeletal muscle tissue, liver tissue, lung tissue, kidney tissue, myocardial tissue, brain tissue, bone marrow, cervix tissue, skin, etc. The sample may be a liquid sample or a liquid extract of a solid sample. In certain cases, the source of the sample may be an organ or tissue, such as a biopsy sample, which may be solubilized by tissue disintegration/cell lysis.

[0339] A wide range of volumes of the fluid sample may be analyzed. In a few exemplary embodiments, the sample volume may be about 0.5 nL, about 1 nL, about 3 nL, about 0.01 μL, about 0.1 μL, about 1 μL, about 5 μL, about 10 μL, about 100 μL, about 1 mL, about 5 mL, about 10 mL, or the like. In some cases, the volume of the fluid sample is between about 0.01 μL and about 10 mL, between about 0.01 μL and about 1 mL, between about 0.01 μL and about 100 μL, or between about 0.1 μL and about 10 μL.

[0340] In some cases, the fluid sample may be diluted prior to use in an assay. For example, in embodiments where the source of UCH-L1 and/or GFAP is a human body fluid (*e.g.*, blood, serum), the fluid may be diluted with an appropriate solvent (*e.g.*, a buffer such as PBS buffer). A fluid sample may be diluted about 1-fold, about 2-fold, about 3-fold, about 4-fold, about 5-fold, about 6-fold, about 10-fold, about 100-fold, or greater, prior to use. In other cases, the fluid sample is not diluted prior to use in an assay.

[0341] In some cases, the sample may undergo pre-analytical processing. Pre-analytical processing may offer additional functionality such as nonspecific protein removal and/or effective yet cheaply implementable mixing functionality. General methods of pre-analytical processing may include the use of electrokinetic trapping, AC electrokinetics, surface acoustic waves, isotachophoresis, dielectrophoresis, electrophoresis, or other pre-

concentration techniques known in the art. In some cases, the fluid sample may be concentrated prior to use in an assay. For example, in embodiments where the source of UCH-L1 and/or GFAP is a human body fluid (e.g., blood, serum), the fluid may be concentrated by precipitation, evaporation, filtration, centrifugation, or a combination thereof. A fluid sample may be concentrated about 1-fold, about 2-fold, about 3-fold, about 4-fold, about 5-fold, about 6-fold, about 10-fold, about 100-fold, or greater, prior to use.

c. Controls

[0342] It may be desirable to include a control sample. The control sample may be analyzed concurrently with the sample from the subject as described above. The results obtained from the subject sample can be compared to the results obtained from the control sample. Standard curves may be provided, with which assay results for the sample may be compared. Such standard curves present levels of marker as a function of assay units, *i.e.*, fluorescent signal intensity, if a fluorescent label is used. Using samples taken from multiple donors, standard curves can be provided for reference levels of the UCH-L1 and/or GFAP in normal healthy tissue, as well as for "at-risk" levels of the UCH-L1 and/or GFAP in tissue taken from donors, who may have one or more of the characteristics set forth above.

[0343] Thus, in view of the above, a method for determining the presence, amount, or concentration of UCH-L1 and/or GFAP in a test sample is provided. The method comprises assaying the test sample for UCH-L1 and/or GFAP by an immunoassay, for example, employing at least one capture antibody that binds to an epitope on UCH-L1 and/or GFAP and at least one detection antibody that binds to an epitope on UCH-L1 and/or GFAP which is different from the epitope for the capture antibody and optionally includes a detectable label, and comprising comparing a signal generated by the detectable label as a direct or indirect indication of the presence, amount or concentration of UCH-L1 and/or GFAP in the test sample to a signal generated as a direct or indirect indication of the presence, amount or concentration of UCH-L1 and/or GFAP in a calibrator. The calibrator is optionally, and is preferably, part of a series of calibrators in which each of the calibrators differs from the other calibrators in the series by the concentration of UCH-L1 and/or GFAP.

12. Kit

[0344] Provided herein is a kit, which may be used for assaying or assessing a test sample for UCH-L1 and/or GFAP or UCH-L1 and/or GFAP fragment. The kit comprises at least one component for assaying the test sample for UCH-L1 and/or GFAP instructions for assaying

the test sample for UCH-L1 and/or GFAP. For example, the kit can comprise instructions for assaying the test sample for UCH-L1 and/or GFAP by immunoassay, e.g., chemiluminescent microparticle immunoassay. Instructions included in kits can be affixed to packaging material or can be included as a package insert. While the instructions are typically written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this disclosure. Such media include, but are not limited to, electronic storage media (*e.g.*, magnetic discs, tapes, cartridges, chips), optical media (e.g., CD ROM), and the like. As used herein, the term "instructions" can include the address of an internet site that provides the instructions.

[0345] The at least one component may include at least one composition comprising one or more isolated antibodies or antibody fragments thereof that specifically bind to UCH-L1 and/or GFAP. The antibody may be a UCH-L1 and/or GFAP capture antibody and/or a UCH-L1 and/or GFAP detection antibody.

[0346] Alternatively or additionally, the kit can comprise a calibrator or control, e.g., purified, and optionally lyophilized, UCH-L1 and/or GFAP, and/or at least one container (e.g., tube, microtiter plates or strips, which can be already coated with an anti-UCH-L1 and/or GFAP monoclonal antibody) for conducting the assay, and/or a buffer, such as an assay buffer or a wash buffer, either one of which can be provided as a concentrated solution, a substrate solution for the detectable label (*e.g.*, an enzymatic label), or a stop solution. Preferably, the kit comprises all components, *i.e.*, reagents, standards, buffers, diluents, etc., which are necessary to perform the assay. The instructions also can include instructions for generating a standard curve.

[0347] The kit may further comprise reference standards for quantifying UCH-L1 and/or GFAP. The reference standards may be employed to establish standard curves for interpolation and/or extrapolation of UCH-L1 and/or GFAP concentrations. The reference standards may include a high UCH-L1 and/or GFAP concentration level, for example, about 100000 pg/mL, about 125000 pg/mL, about 150000 pg/mL, about 175000 pg/mL, about 200000 pg/mL, about 225000 pg/mL, about 250000 pg/mL, about 275000 pg/mL, or about 300000 pg/mL; a medium UCH-L1 and/or GFAP concentration level, for example, about 25000 pg/mL, about 40000 pg/mL, about 45000 pg/mL, about 50000 pg/mL, about 55000 pg/mL, about 60000 pg/mL, about 75000 pg/mL or about 100000 pg/mL; and/or a low UCH-L1 and/or GFAP concentration level, for example, about 1 pg/mL, about 5 pg/mL, about 10 pg/mL, about 12.5 pg/mL, about 15 pg/mL, about 20 pg/mL, about 25 pg/mL, about 30 pg/mL, about 35 pg/mL, about 40 pg/mL, about 45 pg/mL, about 50 pg/mL, about 55 pg/mL,

about 60 pg/mL, about 65 pg/mL, about 70 pg/mL, about 75 pg/mL, about 80 pg/mL, about 85 pg/mL, about 90 pg/mL, about 95 pg/mL, or about 100 pg/mL.

[0348] Any antibodies, which are provided in the kit, such as recombinant antibodies specific for UCH-L1 and/or GFAP, can incorporate a detectable label, such as a fluorophore, radioactive moiety, enzyme, biotin/avidin label, chromophore, chemiluminescent label, or the like, or the kit can include reagents for labeling the antibodies or reagents for detecting the antibodies (e.g., detection antibodies) and/or for labeling the analytes (e.g., UCH-L1 and/or GFAP) or reagents for detecting the analyte (e.g., UCH-L1 and/or GFAP). The antibodies, calibrators, and/or controls can be provided in separate containers or pre-dispensed into an appropriate assay format, for example, into microtiter plates.

[0349] Optionally, the kit includes quality control components (for example, sensitivity panels, calibrators, and positive controls). Preparation of quality control reagents is well-known in the art and is described on insert sheets for a variety of immunodiagnostic products. Sensitivity panel members optionally are used to establish assay performance characteristics, and further optionally are useful indicators of the integrity of the immunoassay kit reagents, and the standardization of assays.

[0350] The kit can also optionally include other reagents required to conduct a diagnostic assay or facilitate quality control evaluations, such as buffers, salts, enzymes, enzyme co-factors, substrates, detection reagents, and the like. Other components, such as buffers and solutions for the isolation and/or treatment of a test sample (e.g., pretreatment reagents), also can be included in the kit. The kit can additionally include one or more other controls. One or more of the components of the kit can be lyophilized, in which case the kit can further comprise reagents suitable for the reconstitution of the lyophilized components.

[0351] The various components of the kit optionally are provided in suitable containers as necessary, e.g., a microtiter plate. The kit can further include containers for holding or storing a sample (e.g., a container or cartridge for a urine, whole blood, plasma, or serum sample). Where appropriate, the kit optionally also can contain reaction vessels, mixing vessels, and other components that facilitate the preparation of reagents or the test sample. The kit can also include one or more instrument for assisting with obtaining a test sample, such as a syringe, pipette, forceps, measured spoon, or the like.

[0352] If the detectable label is at least one acridinium compound, the kit can comprise at least one acridinium-9-carboxamide, at least one acridinium-9-carboxylate aryl ester, or any combination thereof. If the detectable label is at least one acridinium compound, the kit also can comprise a source of hydrogen peroxide, such as a buffer, solution, and/or at least one

basic solution. If desired, the kit can contain a solid phase, such as a magnetic particle, bead, test tube, microtiter plate, cuvette, membrane, scaffolding molecule, film, filter paper, disc, or chip.

[0353] If desired, the kit can further comprise one or more components, alone or in further combination with instructions, for assaying the test sample for another analyte, which can be a biomarker, such as a biomarker of traumatic brain injury or disorder.

a. Adaptation of Kit and Method

[0354] The kit (or components thereof), as well as the method for assessing or determining the concentration of UCH-L1 and/or GFAP in a test sample by an immunoassay as described herein, can be adapted for use in a variety of automated and semi-automated systems (including those wherein the solid phase comprises a microparticle), as described, e.g., U.S. Patent No. 5,063,081, U.S. Patent Application Publication Nos. 2003/0170881, 2004/0018577, 2005/0054078, and 2006/0160164 and as commercially marketed e.g., by Abbott Laboratories (Abbott Park, IL) as Abbott Point of Care (i-STAT® or i-STAT Alinity, Abbott Laboratories) as well as those described in U.S. Patent Nos. 5,089,424 and 5,006,309, and as commercially marketed, *e.g.*, by Abbott Laboratories (Abbott Park, IL) as ARCHITECT® or the series of Abbott Alinity devices.

[0355] Some of the differences between an automated or semi-automated system as compared to a non-automated system (e.g., ELISA) include the substrate to which the first specific binding partner (e.g., analyte antibody or capture antibody) is attached (which can affect sandwich formation and analyte reactivity), and the length and timing of the capture, detection, and/or any optional wash steps. Whereas a non-automated format such as an ELISA may require a relatively longer incubation time with sample and capture reagent (e.g., about 2 hours), an automated or semi-automated format (*e.g.*, ARCHITECT® and any successor platform, Abbott Laboratories) may have a relatively shorter incubation time (e.g., approximately 18 minutes for ARCHITECT®). Similarly, whereas a non-automated format such as an ELISA may incubate a detection antibody such as the conjugate reagent for a relatively longer incubation time (*e.g.*, about 2 hours), an automated or semi-automated format (e.g., ARCHITECT® and any successor platform) may have a relatively shorter incubation time (e.g., approximately 4 minutes for the ARCHITECT® and any successor platform).

[0356] Other platforms available from Abbott Laboratories include, but are not limited to, AxSYM®, IMx® (see, e.g., U.S. Patent No. 5,294,404, which is hereby incorporated by

reference in its entirety), PRISM®, EIA (bead), and Quantum™ II, as well as other platforms. Additionally, the assays, kits, and kit components can be employed in other formats, for example, on electrochemical or other hand-held or point-of-care assay systems. As mentioned previously, the present disclosure is, for example, applicable to the commercial Abbott Point of Care (i-STAT®, Abbott Laboratories) electrochemical immunoassay system that performs sandwich immunoassays. Immunosensors and their methods of manufacture and operation in single-use test devices are described, for example in, U.S. Patent No. 5,063,081, U.S. Patent App. Publication Nos. 2003/0170881, 2004/0018577, 2005/0054078, and 2006/0160164, which are incorporated in their entireties by reference for their teachings regarding same.

[0357] In particular, with regard to the adaptation of an assay to the i-STAT® system, the following configuration is preferred. A microfabricated silicon chip is manufactured with a pair of gold amperometric working electrodes and a silver-silver chloride reference electrode. On one of the working electrodes, polystyrene beads (0.2 mm diameter) with immobilized capture antibody are adhered to a polymer coating of patterned polyvinyl alcohol over the electrode. This chip is assembled into an i-STAT® cartridge with a fluidics format suitable for immunoassay. On a portion of the silicon chip, there is a specific binding partner for UCH-L1 and/or GFAP, such as one or more UCH-L1 and/or GFAP antibodies (one or more monoclonal/polyclonal antibody or a fragment thereof, a variant thereof, or a fragment of a variant thereof that can bind UCH-L1 and/or GFAP) or one or more anti-UCH-L1 and/or GFAP DVD-Igs (or a fragment thereof, a variant thereof, or a fragment of a variant thereof that can bind UCH-L1 and/or GFAP and/or GFAP), either of which can be detectably labeled. Within the fluid pouch of the cartridge is an aqueous reagent that includes p-aminophenol phosphate.

[0358] In operation, a sample from a subject suspected of suffering from TBI is added to the holding chamber of the test cartridge, and the cartridge is inserted into the i-STAT® reader. A pump element within the cartridge pushes the sample into a conduit containing the chip. The sample is brought into contact with the sensors allowing the enzyme conjugate to dissolve into the sample. The sample is oscillated across the sensors to promote formation of the sandwich of approximately 2-12 minutes. In the penultimate step of the assay, the sample is pushed into a waste chamber and wash fluid, containing a substrate for the alkaline phosphatase enzyme, is used to wash excess enzyme conjugate and sample off the sensor chip. In the final step of the assay, the alkaline phosphatase label reacts with p-aminophenol phosphate to cleave the phosphate group and permit the liberated p-aminophenol to be

electrochemically oxidized at the working electrode. Based on the measured current, the reader is able to calculate the amount of UCH-L1 and/or GFAP in the sample by means of an embedded algorithm and factory-determined calibration curve.

[0359] The methods and kits as described herein necessarily encompass other reagents and methods for carrying out the immunoassay. For instance, encompassed are various buffers such as are known in the art and/or which can be readily prepared or optimized to be employed, *e.g.*, for washing, as a conjugate diluent, and/or as a calibrator diluent. An exemplary conjugate diluent is ARCHITECT® conjugate diluent employed in certain kits (Abbott Laboratories, Abbott Park, IL) and containing 2-(N-morpholino)ethanesulfonic acid (MES), a salt, a protein blocker, an antimicrobial agent, and a detergent. An exemplary calibrator diluent is ARCHITECT® human calibrator diluent employed in certain kits (Abbott Laboratories, Abbott Park, IL), which comprises a buffer containing MES, other salt, a protein blocker, and an antimicrobial agent. Additionally, as described in U.S. Patent Application No. 61/142,048 filed December 31, 2008, improved signal generation may be obtained, *e.g.*, in an i-STAT® cartridge format, using a nucleic acid sequence linked to the signal antibody as a signal amplifier.

[0360] While certain embodiments herein are advantageous when employed to assess disease, such as traumatic brain injury, the assays and kits also optionally can be employed to assess UCH-L1 and/or GFAP in other diseases, disorders, and conditions as appropriate.

[0361] The method of assay also can be used to identify a compound that ameliorates diseases, such as traumatic brain injury. For example, a cell that expresses UCH-L1 and/or GFAP can be contacted with a candidate compound. The level of expression of UCH-L1 and/or GFAP in the cell contacted with the compound can be compared to that in a control cell using the method of assay described herein.

[0362] The present invention has multiple aspects, illustrated by the following non-limiting examples.

13. Examples

[0363] It will be readily apparent to those skilled in the art that other suitable modifications and adaptations of the methods of the present disclosure described herein are readily applicable and appreciable, and may be made using suitable equivalents without departing from the scope of the present disclosure or the aspects and embodiments disclosed herein. Having now described the present disclosure in detail, the same will be more clearly understood by reference to the following examples, which are merely intended only to

illustrate some aspects and embodiments of the disclosure, and should not be viewed as limiting to the scope of the disclosure. The disclosures of all journal references, U.S. patents, and publications referred to herein are hereby incorporated by reference in their entireties.

[0364] The present invention has multiple aspects, illustrated by the following non-limiting examples.

Example 1

i-STAT® UCH-L1 Assay

[0365] The i-STAT® UCH-L1 assay was used in a TBI patient population study. Monoclonal antibody pairs, such as Antibody A as a capture monoclonal antibody and Antibody B and C as a detection monoclonal antibody, were used. Antibody A is an exemplary anti-UCH-L1 antibody that was internally developed at Abbott Laboratories (Abbott Park, IL). Antibody B and C recognize different epitopes of UCH-L1 and enhance the detection of antigen in the sample that were developed by Banyan Biomarkers (Alachua, Florida). Other antibodies that were internally developed at Abbott Laboratories (Abbott Park, IL) also show or are expected to show similar enhancement of signal when used together as capture antibodies or detection antibodies, in various combinations. The UCH-L1 assay design was evaluated against key performance attributes. The cartridge configuration was Antibody Configuration: Antibody A (Capture Antibody)/Antibody B+C (Detection Antibody); Reagent conditions: 0.8% solids, 125 µg /mL Fab Alkaline Phosphatase cluster conjugate; and Sample Inlet Print: UCH-L1 standard. The assay time was 10-15 min (with 7-12 min sample capture time).

Example 2

i-STAT® GFAP Assay

[0366] The i-STAT® GFAP assay was used in a TBI patient population study. Monoclonal antibody pairs, such as Antibody A as a capture monoclonal antibody and Antibody B as a detection monoclonal antibody, were used. Antibody A and Antibody B are exemplary anti-GFAP antibodies that were internally developed at Abbott Laboratories (Abbott Park, IL). The GFAP assay design was evaluated against key performance attributes. The cartridge configuration was Antibody Configuration: Antibody A (Capture Antibody)/Antibody B (Detection Antibody); Reagent conditions: 0.8% solids, 250 µg/mL Fab Alkaline Phosphatase cluster conjugate; and Sample Inlet Print: GFAP specific. The assay time was 10-15 min (with 7-12 min sample capture time).

Example 3

Development of a Multi-Modality Classification Scheme for Traumatic Brain Injury

[0367] The goal of this study was to develop a classification scheme for brain injury that indicates the nature (type) and severity of injury. For example, serum biomarkers revealed cell type. Trauma patients were divided into three groups for analysis: only brain injured, only non-brain injured, and combined injury. Brain injured and non-brain injured trauma groups were compared to each other and to the combination of brain/nonbrain trauma. These trauma groups were compared to non-trauma controls. CSF from trauma patients was compared to CSF from non-trauma patients. A secondary goal was to determine whether any of the measures, alone or in combination, had utility as a predictor of clinical outcome after TBI.

[0368] An objective multi-modality classification scheme and outcome measure for traumatic brain injury was developed based on several measures: 1) blood-based biomarkers; 2) physiologic measures and evaluation; and 3) radiographic measures (CT and 3T MRI). Blood-based biomarkers can indicate which cell types are damaged (e.g., glial vs. neuronal) and radiography can detect structural changes.

[0369] **Study Site:** Trauma patient were recruited at Hennepin County Medical Center (HCMC) in the state of Minnesota. Participants included trauma patients of all ages presenting to the HCMC Emergency Department (ED), trauma bay, or as direct transfer to neurosurgery. Trauma patients were excluded if they had a major psychiatric or neurologic disorder, were developmentally abnormal, or were prisoners. Subjects were identified by searching medical records for all trauma admissions and cross-checking with the American College of Surgeons trauma registry utilized in the hospital.

[0370] All trauma patients were recruited for screening at the time of presentation and underwent: 1) a standardized (templated) history and physical examination; 2) analysis of serum biomarkers if blood is drawn for other indications; 3) radiographic study as clinically indicated; 4) follow-up as clinically indicated with 1)-3); 5) pathologic specimen analysis in patients going to the operating room only; 6) CSF analysis in patients receiving ventriculostomy catheters only; 7) brain tissue oxygenation analysis in patients receiving Licox only; and 8) outcome assessment in the TBI center as clinically indicated. At the time of admission, the potential participants underwent a 24 hour screening process (Table 4) before providing informed consent.

Table 4 Screening Assessments

	Adult	Pediatric
All	Surgery-Trauma History and Physical	
	Selections from Neurosurgery-Trauma History and Physical	
Awake	SCAT3: SAC, SSS-C	Child SCAT3: SAC-C, SSS-C
OR	Pathogenic Specimen	
VC	CSF Analysis	
Licox	Brain Tissue Oxygenation	

OR: Patients going to the operating room; VC: Patients receiving ventriculostomy catheters; Licox: Patients receiving Licox

[0371] In addition, patients who consented and controls (age and gender matched) undergone the above plus the following additional studies: 1 genomic, serum, and CSF; and 2) 3T MRI in select circumstances (serum markers in the test group; normal markers in the control group). Trauma patients included the full spectrum ranging from non-brain injured, CT-negative to structurally brain injured, requiring surgery. A patients and controls were recruited over approximately 15 months. Surviving trauma subjects were followed until they were discharged from HCMC services. Subjects evaluated in the ER and released were invited for research follow-up.

[0372] The screening process included a standardized and templated medical history and physical examination. The template was the current "Surgery-Trauma History and Physical" template in EPIC with one additional question that asks if the patient suffered a head trauma. If the patient did, three sections automatically dropped down for additional information. The first was information from the Neurosurgery trauma history and physical template, including subarachnoid grade, hemorrhage grade, intracerebral hemorrhage, and social history (level of education, employment, living arrangements, and ethnicity). The final two were standardized brain injury assessment tools: the Standardized Assessment of Concussion (SAC) and the Symptom Severity Score (SSS). Pediatric versions of these assessments were available and used when indicated. Additionally, the loss of consciousness question already included in the "Surgery Trauma History and Physical" template was copied into this drop down section with a subset of questions that provided a more clear understanding of the loss of consciousness event and the patient's current orientation. The most clinically accurate assessment taken during the first 24 hours of admission was used for future data analysis.

[0373] **Specimen Collection and Handling**. Up to 40 mL (approximately 3 tablespoons) of blood were obtained at each specimen collection. 2 tubes of serum and 2 tubes of plasma were drawn at Encounters 1, 2 4, and 5. At Encounter 3, 2 tubes of serum, 1 tube of plasma and 1 or 2 tubes of whole blood were drawn. These study specimens were processed, aliquoted, frozen, and shipped to Abbott Laboratories for biomarker testing and storage. Sample aliquots were sent to testing sites for additional TBI biomarker testing. A specimen was considered unevaluable for the study if: it contained insufficient volume to perform necessary measurements, it was grossly hemolyzed, lipemic, or icteric; it was not collected in the proper type of collection tube; it was not properly labeled; or it was not properly stored by the collection site or at Abbott Laboratories.

[0374] Serum specimens were obtained via blood draw. If a blood draw was obtained at the time of admission for clinical purposes, additional specimen was obtained and retained for research purposes. If blood was not drawn for clinical purposes, trained research personnel drawn the blood required for research. The blood was drawn through venipuncture unless a central venous access was required by the standard of care, in which case the blood was withdrawn via that access. The first blood draw was taken upon admission, the second 3-6 hours after the first, and the third was taken at 24 hours after the trauma. Discovery efforts were also ongoing to find genetic markers of susceptibility to TBI or predictive markers of TBI. At each time point, 40 mL (less than 3 tablespoons) of blood was collected: 20 mL of serum (2 tubes) and 20 mL of plasma (2 tubes). During Encounter 1, only 2 tubes of serum and 1 tube of plasma were collected for blood biomarker analysis. This whole blood collection was 6.0 mL in a whole blood tube. If a patient was enrolled at Encounter 2 instead of Encounter 1, they had 2 tubes of serum and 1 tube of plasma collected for blood biomarker analysis. This whole blood collection was 6.0 mL in a whole blood tube.

[0375] The amount of blood drawn was limited according to the NINOS standardized table (Table 5). The number of attempts to draw blood was limited for children under the age of seven to two attempts. In the case that the NINOS standardized table did not allow enough blood to be drawn for the study or there were two failed attempts to draw blood in a child patient, access to leftover blood samples drawn under the clinical standard of care was requested for complete biomarker analysis in this study. This blood draw allowed for the analysis of up to 390 blood-based biomarkers related to traumatic brain injuries.

Table 5 Maximum Allowable Total Blood Draw Volumes

Body Wt (Kg)	Body Wt (lbs)	Total blood volume (mL)	Maximum allowable volume (mL) in one blood draw (= 2.5% of total blood volume)	Maximum volume (clinical + research) (mL) in a 30-day period	Minimum Hgb required at time of blood draw	Minimum Hgb required at time of blood draw if subject has respiratory/CV compromise
1	2.2	100	2.5	5	7.0	9.0-10.0
2	4.4	200	5	10	7.0	9.0-10.0
3	6.3	240	6	12	7.0	9.0-10.0
4	8.8	320	8	16	7.0	9.0-10.0
5	11	400	10	20	7.0	9.0-10.0
6	13.2	480	12	24	7.0	9.0-10.0
7	15.4	560	14	28	7.0	9.0-10.0
8	17.6	640	16	32	7.0	9.0-10.0
9	19.8	720	18	36	7.0	9.0-10.0
10	22	800	20	40	7.0	9.0-10.0
11-15	24-33	880-1200	22-30	44-60	7.0	9.0-10.0
16-20	35-44	1280-1600	32-40	64-80	7.0	9.0-10.0
21-25	46-55	1680-2000	42-50	84-100	7.0	9.0-10.0
26-30	57-66	2080-2400	52-60	104-120	7.0	9.0-10.0
31-35	68-77	2480-2800	62-70	124-140	7.0	9.0-10.0
36-40	79-88	2880-3200	72-80	144-160	7.0	9.0-10.0
41-45	90-99	3280-3600	82-90	164-180	7.0	9.0-10.0
46-50	101-110	3680-4000	92-100	184-200	7.0	9.0-10.0
51-55	112-121	4080-4400	102-110	204-220	7.0	9.0-10.0
56-60	123-132	4480-4800	112-120	224-240	7.0	9.0-10.0
61-65	134-143	4880-5200	122-130	244-260	7.0	9.0-10.0
66-70	145-154	5280-5600	132-140	264-280	7.0	9.0-10.0
71-75	156-165	5680-6000	142-150	284-300	7.0	9.0-10.0
76-80	167-176	6080-6400	152-160	304-360	7.0	9.0-10.0
81-85	178-187	6480-6800	162-170	324-340	7.0	9.0-10.0
86-90	189-198	6880-7200	172-180	344-360	7.0	9.0-10.0
91-95	200-209	7280-7600	182-190	364-380	7.0	9.0-10.0
96-100	211-220	7680-8000	192-200	384-400	7.0	9.0-10.0

[0376] In addition to the initial physical exam, those patients that were sent to the operating room undergone pathologic specimen analysis, those patients that received Licox had brain tissue oxygenation information recorded, and those patients that received ventriculostomy catheters had CSF collected for analysis. In order to analyze the CSF, 5.0 mL was collected at the same intervals that blood was drawn. Radiographic studies were performed in accordance with the standard of care. None of the assessments performed during the screening processes were analyzed with the rest of the data until informed consent was obtained. If the patient did not ultimately consent to research, the specimens, and initial assessments were discarded.

[0377] After the participants were discharged, the patients' medical records were accessed for information about the clinical course, including time spent in the ED, any surgeries or other neuromonitoring methods used, and the acute care outcome evaluation. If the patient spent time in the ICU, information was extracted from that time period as well, including data from Moberg monitors and daily therapeutic intensity level.

[0378] Trauma patients were divided into three groups for analysis: only brain injured, only non-brain injured, and combined injury. Two age- and gender-matched control groups were included in this study and recruited from the ED: non-trauma and CSF controls. Non-trauma controls were those who did not experience any trauma, and this group composed largely of family and friends of the patients admitted for brain injury. Both control groups were consented to undergo a single intensive assessment that included a blood draw and cognitive, neurological, and quality of life assessments (SAC, NOS-TBI, QoLABI). Patients receiving elective ventriculostomy or lumbar drain catheters were (pre-operatively) consented to be a part of the CSF control group. 5 mL of CSF was collected from the ventriculostomy catheter of patients in this control group for comparison with the CSF collected from the portion of the study group that received a ventriculostomy catheter as a part of their standard of care. The CSF control group was also offered the chance to participate in the same intensive assessment as the other two controls groups that included a blood draw and a 3T MRI scan.

[0379] **Follow-Up:** All patients that consented to participate in the follow-up portion of the study were asked to return to the hospital. Patients who returned were seen in the TBI Outpatient Clinic at 2 weeks, 4 weeks, months, 6 months, and 1 year. If they did not have a scheduled appointment at the TBI Outpatient Clinic, they were scheduled a time to come into the Brain Injury Research Lab (PL.610) at those time points. Table 6 provides a timeline for each of the assessments. Blood draws for biomarker analysis were done at each of the five follow-up time points in the same method, as described above. The outcome assessment battery listed in Tables 7 and 8 were completed at 3 months, 6 months, and one year. Radiographic scans that were a part of the standard of care were accessed through the participant's medical records, but select consented participants and controls also underwent 3T MRI scans at 2 weeks and 6 months after their brain injury. Each MRI examination took approximately one hour and included the following pulse sequences: (1) Sagittal short TR localizer, (2) Axial Fse, (3) Axial FLAIR, (4) Axial SWI, (5) Axial T2* imaging. In the case that patients were not able to come into the hospital for follow-up, they were contacted via phone at three months and one year after their injury to complete the BT ACT, which was a 15-20 minute cognitive assessment designed to be administered over the phone.

Table 6 Outcome Timeline

	Blood Draw	3T MRI	CSF Collection	CT Scan	Assessments	Total Time (minutes)

ADM	X		X (if indicated)			10
2 weeks	X	X				70
4 weeks	X					10
3 months	X				X	70
6 months	X	X				70
1 year	X	X		X*	X	160 (130 without CT)

Table 7 Outcome Assessments – Not Finalized

		Adult	Pediatric
Outcome	All	GOS and GOSE	Pediatric GOSE
	Awake	SCAT3: SSS and SAC	Child SCAT3: Child & Parent Report; SAC-C
		GOAT	COAT
		Duration of Amnesia	Duration of Amnesia
		NOS-TBI	NOS-TBI
		Quality of Life: - MPAI-4	Quality of Life: - Neuropsychiatric Rating Schedule - Pediatric Quality of Life Inventory (For Child and for Proxy)
	Not Awake	CRS-R (Just Brain Stem Reflex Grid?)	

Table 8 Possible Infant Assessments

Name	Age	Time	Description
Bayley III, BSID*	0-3.5	30-90	Cognitive, language (receptive and expressive) and motor development Most commonly used in test for this age range
BITSEA*	1-3	7-12	Parent perception of Social and Emotional behavior 17 items of 42 are for autism, so may be able to be made shorter
CBCL	1.5-5	25-30	Parent perception of performance on Activities, Social, and School performance
MSEL*	1 3 5	15 25-35 40-60	Cognitive and Motor Ability (Gross Motor, Visual Reception, Fine Motor, Language Mostly for readiness for school
Shape School*	3-6	45-75	Inhibition and Switching Processes: Emerging Executive Functions
Trails-Preschool*	2-6	5-10	Neuropsychological Function: Psychomotor speed, Complex Attention, Executive Function Advanced Trail Making Test

*Requested Access

[0380] Statistical Analysis Plan. Biomarker data was analyzed by examining maximum concentration draw for each biomarker per patient, or by time from incident buckets, or both. To address the primary objective of determining associations between biomarker

concentrations in blood and clinical neurological and magnetic resonance imaging data, multiple analyses was employed. Principal components analysis was used to examine which biomarkers may be explaining the same variance, or if a biomarker was contributing very little variance. The biomarkers were used in a logistic regression analysis, and some biomarkers were excluded based on the results from the principal components analysis, and clinical input. A significance level of 0.05 was used for the logistic regression analysis. ROC analysis was also used to examine the predictive ability of each biomarker in determining MRI status or neurological testing outcomes, for this set of data.

Example 4

[0381] To evaluate blood levels of biomarkers such as GFAP and UCH-L1 in predicting computed tomography (CT)-positive TBI, blood samples were collected within about 6 hours (e.g., within 0.5 hours, 1.0 hours, 1.5 hours, 2.0 hours, 2.5 hours, 3.0 hours, 3.5 hours, 4.0 hours, 4.5 hours, 5.0 hours and 5.5 hours) of injury from 78 trauma subjects who underwent a head CT scan. Biomarker concentrations of subjects with positive (Marshall classification ≥ 2; n=34) or negative (Marshall classification =1; n=44) CT scans were compared. In addition, biomarker concentrations of subjects in the trauma group were compared with 35 nontrauma control subjects. Median levels of UCH-L1 for control versus trauma groups were 0.074 and 0.780 ng/mL. Corresponding median levels of GFAP were 0.009 and 0.109 ng/mL. Blood concentrations of both biomarkers were significantly different between the groups (both $P<0.001$). In trauma subjects, GFAP ($P<0.001$) and UCH-L1 ($P=0.008$) were higher in subjects with positive CT scans compared to those with negative CT scans. These findings indicate that concentrations of UCH-L1 and GFAP were increased in patients with TBI. Furthermore, these preliminary data suggest higher levels of GFAP and UCH-L1 predict a positive CT scan with high sensitivity, suggesting that these biomarkers may have clinical utility in the management of brain injury.

[0382] UCH-L1 and GFAP levels in samples taken from human subjects within about 2 hours of suspected injury were measured. FIGS. 1 and 2 show the levels and the CT scan results for the subjects. FIGS. 3 and 4 show the levels and the GCS Score results for the subjects. FIGS. 1 and 3 show the UCH-L1 levels and FIGS. 2 and 4 show the GFAP levels. Table 9 shows the results of samples taken at 10 minutes, 12 minutes, and 20 minutes after injury. Table 10 shows the levels in control subjects.

Table 9

Time of Blood Draw (minutes)	CT Status	GCS Score	UCH-L1 (pg/mL)	GFAP (pg/mL)
10	Positive	Mild	1669	108
12	Negative	Mild	3285	54
20	Positive	Severe	2919	1809

Table 10

Assay	N	Mean	Median	Min	Max	Interquartile Range (IQR: Q3 – Q1)
GFAP (pg/mL)	36	11.7	9	1	42	13 – 6
UCH-L1 (pg/mL)	36	88.2	73.5	0	371	112.5 – 44

[0383] Sensitivity was determined by the test: TP/T_{TBI} and specificity was determined by the test: $TN/T_{NOT\ TBI}$. PPV was determined by $TP/T_{POSITIVE}$. NPV was determined by $TN/T_{NEGATIVE}$. Accuracy was determined by $(TP + TN)/T_{ALL\ SUBJECTS}$.

[0384] FIGS. 5 and 6 show box plots of UCH-L1 levels (FIG. 5) and GFAP levels (FIG. 6) in human subjects correlated with their CT scan results (positive vs. negative) compared with levels in control human subjects. FIGS. 7 and 8 show ROC analysis of UCH-L1 levels (FIG. 7) and GFAP levels (FIG. 8) correlated with CT status (positive vs. negative CT scan result). FIG. 9 shows ROC analysis of the combination of UCH-L1 levels and GFAP levels correlated with CT status (positive vs. negative CT scan result). Tables 11 and 12 shows the sensitivities and specificities of using UCH-L1 cut-off levels (Table 11), GFAP cut-off levels (Table 11), or a combination of UCH-L1 cut-off levels and GFAP cut-off levels (Table 12) to predict a positive CT scan result.

Table 11 CT Scan – Single Biomarker Reference Levels Analysis

Biomarker	Levels (pg/mL)	Sensitivity	Specificity
UCH-L1	854	83.33%	69.57%
	688	91.67%	68.12%
	78	91.67%	30.43%
GFAP	459	66.67%	98.55%
	193	75.00%	97.10%
	181.9	83.33%	97.10%
	108	91.67%	91.30%
	34	91.67%	71.01%
	33	100.00%	68.12%
	30	100.00%	66.67%
	11	100.00%	31.88%

Table 12 CT Scan – Both Biomarkers Reference Levels Analysis

UCH-L1 Levels (pg/mL)	GFAP Levels (pg/mL)	Sensitivity	Specificity
900	100	66.67%	92.75%
600	100	91.67%	92.75%
400	100	91.67%	91.30%
100	150	83.33%	95.65%
100	100	91.67%	91.30%
100	400	66.67%	98.55%

[0385] FIGS. 10 and 11 show box plots of UCH-L1 levels (FIG. 10) and GFAP levels (FIG. 11) in human subjects correlated with their GCS Score results (mild vs. moderate/severe TBI) compared with levels in control human subjects. FIGS. 12 and 13 show ROC analysis of UCH-L1 levels (FIG. 12) and GFAP levels (FIG. 13) correlated with GCS Score results (mild vs. moderate/severe TBI). FIG. 14 shows ROC analysis of the combination of UCH-L1 levels and GFAP levels correlated with GCS Score results (mild vs. moderate/severe TBI). Tables 13 and 14 shows the sensitivities and specificities of using UCH-L1 cut-off levels (Table 13), GFAP cut-off levels (Table 13), or a combination of both UCH-L1 cut-off levels and GFAP cut-off levels (Table 14) to predict moderate/severe TBI based on GCS scores.

Table 13 GCS Score – Single Biomarker Reference Levels Analysis

Biomarker	Levels (pg/mL)	Sensitivity	Specificity
UCH-L1	3019	70.00%	85.92%
	2919	80.00%	84.51%
	856	90.00%	69.01%
	305	100.00%	54.93%
	78	100.00%	30.99%
GFAP	36	70.00%	69.01%
	28	80.00%	60.56%
	23	90.00%	59.16%
	12	90.00%	35.21%
	11	90.00%	29.58%

Table 14 GCS Score – Both Biomarkers Reference Levels Analysis

UCH-L1 Levels (pg/mL)	GFAP Levels (pg/mL)	Sensitivity	Specificity
78	12	90.00%	46.48%
305	12	90.00%	57.75%
305	20	90.00%	61.97%
305	30	70.00%	67.61%
856	20	80.00%	71.83%

[0386] These results shown in the above tables demonstrate the unexpected finding that UCH-L1 and GFAP levels can be assessed as early as minutes within head injury.

[0387] It is understood that the foregoing detailed description and accompanying examples are merely illustrative and are not to be taken as limitations upon the scope of the invention, which is defined solely by the appended claims and their equivalents.

[0388] Various changes and modifications to the disclosed embodiments will be apparent to those skilled in the art. Such changes and modifications, including without limitation those relating to the chemical structures, substituents, derivatives, intermediates, syntheses, compositions, formulations, or methods of use of the invention, may be made without departing from the spirit and scope thereof.

[0389] For reasons of completeness, various aspects of the invention are set out in the following numbered clauses:

[0390] Clause 1. A method of aiding in the diagnosis and evaluation of a human subject that has sustained or may have sustained an injury to the head, the method comprising: a) performing an assay on a sample obtained from the subject within about 2 hours after a suspected injury to the head to measure or detect a level of an early biomarker in the sample, said early biomarker comprising ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), glial fibrillary acidic protein (GFAP), or a combination thereof; and b) determining whether the subject has sustained a mild or a moderate, severe, or moderate to severe traumatic brain injury (TBI), wherein the subject is determined as having (1) a moderate, severe, moderate to severe traumatic brain injury when the level of the early biomarker in the sample is higher than a reference level of the early biomarker or (2) a mild traumatic brain injury when the level of the early biomarker in the sample is lower than a reference level of the early biomarker.

[0391] Clause 2. The method of clause 1, wherein the subject has received a Glasgow Coma Scale score before or after the assay is performed.

[0392] Clause 3. The method of clause 2, wherein the subject is suspected as having moderate, severe, or moderate to severe traumatic brain injury based on the Glasgow Coma Scale score.

[0393] Clause 4. The method of clause 3, wherein the reference level is correlated with subjects having moderate to severe traumatic brain injury.

[0394] Clause 5. The method of clause 4, wherein the reference level is correlated with a Glasgow Coma Scale score of 3-12.

[0395] Clause 6. The method of clause 2, wherein the subject is suspected as having mild traumatic brain injury based on the Glasgow Coma Scale score.

[0396] Clause 7. The method of clause 6, wherein the reference level is correlated with subjects having mild traumatic brain injury.

[0397] Clause 8. The method of clause 7, wherein the reference level is correlated with a Glasgow Coma Scale score of 13-15.

[0398] Clause 9. The method of clause 1, wherein the reference level is correlated with control subjects that have not sustained a head injury.

[0399] Clause 10. The method of clause 9, wherein the reference level for GFAP is about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL.

[0400] Clause 11. The method of clause 10, wherein the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL.

[0401] Clause 12. The method of clause 9, wherein the reference level for UCH-L1 is about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL.

[0402] Clause 13. The method of clause 12, wherein the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL.

[0403] Clause 14. The method of any one of clauses 1 to 9, wherein the reference level is (a) determined by an assay having a sensitivity of between at least about 65% to 100% and a specificity of between at least about 30% to 100%; (b) determined by an assay having a sensitivity of at least about 99% and a specificity of at least about 75%; (c) between at least about 5 pg/mL to about 3500 pg/mL; or (d) between at least about 10 pg/mL to about 1000 pg/mL.

[0404] Clause 15. The method of any one of clauses 1 to 9 and 14, wherein the reference level for UCH-L1 is between at least about 70 pg/mL to about 3500 pg/mL and the reference level for GFAP is between at least about 5 pg/mL to about 2000 pg/mL.

[0405] Clause 16. The method of any one of clauses 1 to 15, wherein the sample is taken within about 5 minutes, within about 10 minutes, within about 12 minutes, within about 15 minutes, within about 20 minutes, within about 30 minutes, within about 60 minutes, or within about 90 minutes after a suspected injury to the head.

[0406] Clause 17. The method of any one of claims 1 to 16, the sample is taken (a) within about 10 minutes after the suspected injury and the reference level of UCH-L1 is at least about 1669 pg/mL; (b) within about 12 minutes after the suspected injury and the reference

level of UCH-L1 is at least about 3285 pg/mL; (c) within about 20 minutes after the suspected injury and the reference level of UCH-L1 is at least about 2919 pg/mL; (d) within about 10 minutes after the suspected injury and the reference level of GFAP is at least about 108 pg/mL; (e) within about 12 minutes after the suspected injury and the reference level of GFAP is at least about 54 pg/mL; (f) within about 20 minutes after the suspected injury and the reference level of GFAP is at least about 1809 pg/mL; (g) within about 10 minutes after the suspected injury and the reference level of UCH-L1 is at least about 1669 pg/mL and the reference level of GFAP is at least about 108 pg/mL; (h) within about 12 minutes after the suspected injury and the reference level of UCH-L1 is at least about 3285 pg/mL and the reference level of GFAP is at least about 54 pg/mL; or (i) within about 20 minutes after the suspected injury and the reference level of UCH-L1 is at least about 2919 pg/mL and the reference level of GFAP is at least about 1809 pg/mL.

[0407] Clause 18. The method of any one of clauses 1 to 17, further comprising treating the subject assessed as having moderate to severe traumatic brain injury with a traumatic brain injury treatment.

[0408] Clause 19. The method of any one of clauses 1 to 18, further comprising monitoring the subject assessed as having mild traumatic brain injury.

[0409] Clause 20. A method of aiding in the determination of whether to perform a head computerized tomography (CT) scan on a human subject that has sustained or may have sustained a suspected injury to the head, the method comprising: a) performing an assay on a sample obtained from the subject within about 2 hours after a suspected injury to the head to measure or detect a level of an early biomarker in the sample, said early biomarker comprising ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), glial fibrillary acidic protein (GFAP), or a combination thereof, in the sample; and b) performing a CT scan on the subject when the level of the early biomarker in the sample is higher than a reference level of the early biomarker and not performing a CT scan on the subject when the level of the early biomarker in the sample is lower than a reference level of the early biomarker.

[0410] Clause 21. The method of clause 20, wherein the subject has received a CT scan before or after the assay is performed.

[0411] Clause 22. The method of clause 21, wherein the subject is suspected of having a traumatic brain injury based on the CT scan.

[0412] Clause 23. The method of any one of clauses 20 to 22, wherein the reference level is correlated with positive head computed tomography.

[0413] Clause 24. The method of claim 20, wherein the reference level is correlated with control subjects that have not sustained a head injury.

[0414] Clause 25. The method of claim 24, wherein the reference level for GFAP is about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL.

[0415] Clause 26. The method of claim 25, wherein the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL.

[0416] Clause 27. The method of claim 26, wherein the reference level for UCH-L1 is about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL.

[0417] Clause 28. The method of claim 27, wherein the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL.

[0418] Clause 29. The method of any one of clauses 20 to 28, wherein the reference level is (a) determined by an assay having a sensitivity of between at least about 65% to 100% and a specificity of between at least about 30% to 100%; (b) determined by an assay having a sensitivity of at least about 99% and a specificity of at least about 75%; (c) between at least about 5 pg/mL to about 3500 pg/mL; or (d) between at least about 10 pg/mL to about 1000 pg/mL.

[0419] Clause 30. The method of any one of clauses 20 to 29, wherein the reference level for UCH-L1 is between at least about 70 pg/mL to about 3500 pg/mL and the reference level for GFAP is between at least about 5 pg/mL to about 2000 pg/mL.

[0420] Clause 31. The method of any one of clauses 20 to 30, wherein the sample is taken within about 5 minutes, within about 10 minutes, within about 12 minutes, within about 15 minutes, within about 20 minutes, within about 30 minutes, within about 60 minutes, or within about 90 minutes after a suspected injury to the head.

[0421] Clause 32. The method of any one of clauses 20 to 31, the sample is taken (a) within about 10 minutes after the suspected injury and the reference level of UCH-L1 is at least about 1669 pg/mL; (b) within about 12 minutes after the suspected injury and the reference level of UCH-L1 is at least about 3285 pg/mL; (c) within about 20 minutes after the suspected injury and the reference level of UCH-L1 is at least about 2919 pg/mL; (d) within about 10 minutes after the suspected injury and the reference level of GFAP is at least about 108 pg/mL; (e) within about 12 minutes after the suspected injury and the reference level of GFAP is at least about 54 pg/mL; (f) within about 20 minutes after the suspected injury and the reference level of GFAP is at least about 1809 pg/mL; (g) within about 10 minutes after

the suspected injury and the reference level of UCH-L1 is at least about 1669 pg/mL and the reference level of GFAP is at least about 108 pg/mL; (h) within about 12 minutes after the suspected injury and the reference level of UCH-L1 is at least about 3285 pg/mL and the reference level of GFAP is at least about 54 pg/mL; or (i) within about 20 minutes after the suspected injury and the reference level of UCH-L1 is at least about 2919 pg/mL and the reference level of GFAP is at least about 1809 pg/mL.

[0422] Clause 33. The method of any one of clauses 1 to 32, wherein measuring the level of UCH-L1 is done by an immunoassay or clinical chemistry assay.

[0423] Clause 34. The method of any one of clauses 1 to 33, wherein measuring the level of UCH-L1 comprises: A. contacting the sample, either simultaneously or sequentially, in any order with: (1) a UCH-L1-capture antibody, which binds to an epitope on UCH-L1 or UCH-L1 fragment to form a UCH-L1-capture antibody-UCH-L1 antigen complex, and (2) a UCH-L1-detection antibody which includes a detectable label and binds to an epitope on UCH-L1 that is not bound by the UCH-L1-capture antibody, to form a UCH-L1 antigen-UCH-L1-detection antibody complex, such that a UCH-L1-capture antibody-UCH-L1 antigen-UCH-L1-detection antibody complex is formed, and B. measuring the amount or concentration of UCH-L1 in the sample based on the signal generated by the detectable label in the UCH-L1-capture antibody-UCH-L1 antigen-UCH-L1-detection antibody complex.

[0424] Clause 35. The method of any one of clauses 1 to 34, wherein measuring the level of GFAP is done by an immunoassay or clinical chemistry assay.

[0425] Clause 36. The method of any one of clauses 1 to 35, wherein measuring the level of GFAP comprises: A. contacting the sample, either simultaneously or sequentially, in any order with: (1) a GFAP-capture antibody, which binds to an epitope on GFAP or GFAP fragment to form a GFAP-capture antibody-GFAP antigen complex, and (2) a GFAP-detection antibody which includes a detectable label and binds to an epitope on GFAP that is not bound by the GFAP-capture antibody, to form a GFAP antigen-GFAP-detection antibody complex, such that a GFAP-capture antibody-GFAP antigen-GFAP-detection antibody complex is formed, and B. measuring the amount or concentration of GFAP in the sample based on the signal generated by the detectable label in the GFAP-capture antibody-GFAP antigen-GFAP-detection antibody complex.

[0426] Clause 37. The method of any one of clauses 1 to 36, wherein the sample is selected from the group consisting of a whole blood sample, a serum sample, a cerebrospinal fluid sample, and a plasma sample.

[0427] Clause 38. The method of any one of clauses 1 to 37, wherein the sample is obtained after the subject sustained an injury to the head caused by physical shaking, blunt impact by an external mechanical or other force that results in a closed or open head trauma, one or more falls, explosions or blasts or other types of blunt force trauma.

[0428] Clause 39. The method of any one of clauses 1 to 38, wherein the sample is obtained after the subject has ingested or been exposed to a chemical, toxin or combination of a chemical and toxin.

[0429] Clause 40. The method of clause 39, wherein the chemical or toxin is fire, mold, asbestos, a pesticide, an insecticide, an organic solvent, a paint, a glue, a gas, an organic metal, a drug of abuse or one or more combinations thereof.

[0430] Clause 41. The method of any one of clauses 1 to 39, wherein the sample is obtained from a subject that suffers from an autoimmune disease, a metabolic disorder, a brain tumor, hypoxia, a virus, meningitis, hydrocephalus or combinations thereof.

[0431] Clause 42. The method of any one of clauses 1 to 41, wherein said method can be carried out on any subject without regard to factors selected from the group consisting of the subject's clinical condition, the subject's laboratory values, the subject's classification as suffering from mild, moderate, severe, or moderate to severetraumatic brain injury, the subject's exhibition of low or high levels of UCH-L1, GFAP or UCH-L1 and GFAP, and the timing of any event wherein said subject may have sustained an injury to the head.

[0432] Clause 41. The method of any one of clauses 1 to 40, wherein the sample is a whole blood sample.

[0433] Clause 42. The method of any one of clauses 1 to 40, wherein the sample is a serum sample.

[0434] Clause 43. The method of any one of clauses 1 to 40, wherein the sample is a plasma sample.

[0435] Clause 44. The method of any one of clauses 41-43, wherein the assay is an immunoassay.

[0436] Clause 45. The method of any one of clauses 41-43, wherein the assay is a clinical chemistry assay.

[0437] Clause 46. The method of any one of clauses 41-43, wherein the assay is a single molecule detection assay.

Clause 47. A method of evaluating a human subject for a head injury, the method comprising:

a) performing an assay on a sample obtained from the subject within about 2 hours after a suspected injury to the head to measure a level of an early biomarker in the sample, said early biomarker comprising ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), glial fibrillary acidic protein (GFAP), or a combination thereof; and

b) determining whether the subject has sustained a mild or a moderate, severe, or moderate to severe traumatic brain injury (TBI), wherein the subject is determined as having (1) a moderate, severe, moderate to severe traumatic brain injury when the level of the early biomarker in the sample is higher than a reference level of the early biomarker or (2) a mild traumatic brain injury when the level of the early biomarker in the sample is lower than a reference level of the early biomarker.

Clause 48. The method of clause 47, wherein the subject has received a Glasgow Coma Scale score before or after the assay is performed.

Clause 49. The method of clause 48, wherein the subject is suspected as having moderate, severe, or moderate to severetraumatic brain injury based on the Glasgow Coma Scale score.

Clause 50. The method of clause 49, wherein the reference level is correlated with subjects having moderate to severe traumatic brain injury.

Clause 51. The method of clause 50, wherein the reference level is correlated with a Glasgow Coma Scale score of 3-12.

Clause 53. The method of clause 48, wherein the subject is suspected as having mild traumatic brain injury based on the Glasgow Coma Scale score.

Clause 54. The method of clause 53, wherein the reference level is correlated with subjects having mild traumatic brain injury.

Clause 55. The method of clause 54, wherein the reference level is correlated with a Glasgow Coma Scale score of 13-15.

Clause 56. The method of clause 47, wherein the reference level is correlated with control subjects that have not sustained a head injury.

Clause 57. The method of clause 56, wherein the reference level for GFAP is about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL.

Clause 58. The method of clause 57, wherein the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL.

Clause 59. The method of clause 56, wherein the reference level for UCH-L1 is about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL.

Clause 60. The method of clause 59, wherein the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL.

Clause 61. The method of any one of clauses 47 to 56, wherein the reference level is (a) determined by an assay having a sensitivity of between at least about 65% to 100% and a specificity of between at least about 30% to 100%; (b) determined by an assay having a sensitivity of at least about 99% and a specificity of at least about 75%; (c) between at least about 5 pg/mL to about 3500 pg/mL; or (d) between at least about 10 pg/mL to about 1000 pg/mL.

Clause 62. The method of any one of clauses 47 to 56 and 61, wherein the reference level for UCH-L1 is from at least about 70 pg/mL to about 3500 pg/mL and the reference level for GFAP is from at least about 5 pg/mL to about 2000 pg/mL.

Clause 63. The method of any one of clauses 47 to 62, wherein the sample is taken within about 5 minutes, within about 10 minutes, within about 12 minutes, within about 15 minutes, within about 20 minutes, within about 30 minutes, within about 60 minutes, or within about 90 minutes after a suspected injury to the head.

Clause 64. The method of any one of clauses 47 to 63, further comprising treating the subject assessed as having moderate to severe traumatic brain injury with a traumatic brain injury treatment.

Clause 65. The method of any one of clauses 47 to 63, further comprising monitoring the subject assessed as having mild traumatic brain injury.

Clause 66. A method of evaluating whether to perform a head computerized tomography (CT) scan on a human subject that has sustained or may have sustained a suspected injury to the head, the method comprising:

performing an assay on a sample obtained from the subject within about 2 hours after a suspected injury to the head to measure a level of an early biomarker in the sample, said

early biomarker comprising ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), glial fibrillary acidic protein (GFAP), or a combination thereof, in the sample; and

performing a CT scan on the subject when the level of the early biomarker in the sample is higher than a reference level of the early biomarker and not performing a CT scan on the subject when the level of the early biomarker in the sample is lower than a reference level of the early biomarker.

Clause 67. The method of clause 66, wherein the subject has received a CT scan before or after the assay is performed.

Clause 68. The method of clause 67, wherein the subject is suspected of having a traumatic brain injury based on the CT scan.

Clause 69. The method of any one of clauses 66 to 67, wherein the reference level is correlated with positive head computed tomography.

Clause 70. The method of clause 66, wherein the reference level is correlated with control subjects that have not sustained a head injury.

Clause 71. The method of clause 70, wherein the reference level for GFAP is about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL.

Clause 72. The method of clause 71, wherein the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL.

Clause 73. The method of clause 72, wherein the reference level for UCH-L1 is about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL.

Clause 74. The method of clause 73, wherein the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL.

Clause 75. The method of any one of clauses 66 to 74, wherein the reference level is (a) determined by an assay having a sensitivity of between at least about 65% to 100% and a specificity of between at least about 30% to 100%; (b) determined by an assay having a sensitivity of at least about 99% and a specificity of at least about 75%; (c) between at least about 5 pg/mL to about 3500 pg/mL; or (d) between at least about 10 pg/mL to about 1000 pg/mL.

Clause 76. The method of any one of clauses 66 to 75, wherein the reference level for UCH-L1 is between at least about 70 pg/mL to about 3500 pg/mL and the reference level for GFAP is between at least about 5 pg/mL to about 2000 pg/mL.

Clause 77. The method of any one of clauses 66 to 76, wherein the sample is taken within about 5 minutes, within about 10 minutes, within about 12 minutes, within about 15 minutes, within about 20 minutes, within about 30 minutes, within about 60 minutes, or within about 90 minutes after a suspected injury to the head.

Clause 78. The method of any one of clauses 47 to 77, wherein measuring the level of UCH-L1 is done by an immunoassay or clinical chemistry assay.

Clause 79. The method of any one of clauses 47 to 78, wherein measuring the level of UCH-L1 comprises:

A. contacting the sample, either simultaneously or sequentially, in any order with:

(1) a UCH-L1-capture antibody, which binds to an epitope on UCH-L1 or UCH-L1 fragment to form a UCH-L1-capture antibody-UCH-L1 antigen complex, and

(2) a UCH-L1-detection antibody which includes a detectable label and binds to an epitope on UCH-L1 that is not bound by the UCH-L1-capture antibody, to form a UCH-L1 antigen-UCH-L1-detection antibody complex,

such that a UCH-L1-capture antibody-UCH-L1 antigen-UCH-L1-detection antibody complex is formed, and

B. measuring the amount or concentration of UCH-L1 in the sample based on the signal generated by the detectable label in the UCH-L1-capture antibody-UCH-L1 antigen-UCH-L1-detection antibody complex.

Clause 80. The method of any one of clauses 47 to 79, wherein measuring the level of GFAP is done by an immunoassay or clinical chemistry assay.

Clause 81. The method of any one of clauses 47 to 80, wherein measuring the level of GFAP comprises:

A. contacting the sample, either simultaneously or sequentially, in any order with:

(1) a GFAP-capture antibody, which binds to an epitope on GFAP or GFAP fragment to form a GFAP-capture antibody-GFAP antigen complex, and

(2) a GFAP-detection antibody which includes a detectable label and binds to an epitope on GFAP that is not bound by the GFAP-capture antibody, to form a GFAP antigen-GFAP-detection antibody complex,
such that a GFAP-capture antibody-GFAP antigen-GFAP-detection antibody complex is formed, and

B. measuring the amount or concentration of GFAP in the sample based on the signal generated by the detectable label in the GFAP-capture antibody-GFAP antigen-GFAP-detection antibody complex.

Clause 82. The method of any one of clauses 47 to 81, wherein the sample is selected from the group consisting of a whole blood sample, a serum sample, a cerebrospinal fluid sample, and a plasma sample.

Clause 83. The method of any one of clauses 47 to 82, wherein the sample is obtained after the subject sustained an injury to the head caused by physical shaking, blunt impact by an external mechanical or other force that results in a closed or open head trauma, one or more falls, explosions or blasts or other types of blunt force trauma.

Clause 84. The method of any one of clauses 47 to 82, wherein the sample is obtained after the subject has ingested or been exposed to a chemical, toxin or combination of a chemical and toxin.

Clause 85. The method of clause 84, wherein the chemical or toxin is fire, mold, asbestos, a pesticide, an insecticide, an organic solvent, a paint, a glue, a gas, an organic metal, a drug of abuse or one or more combinations thereof.

Clause 86. The method of any one of clauses 47 to 82, wherein the sample is obtained from a subject that suffers from an autoimmune disease, a metabolic disorder, a brain tumor, hypoxia, a virus, meningitis, hydrocephalus or combinations thereof.

Clause 87. The method of any one of clauses 47 to 86, wherein said method can be carried out on any subject without regard to factors selected from the group consisting of the subject's clinical condition, the subject's laboratory values, the subject's classification as suffering from mild, moderate to severe traumatic brain injury, the subject's exhibition of low or high levels of UCH-L1, GFAP or UCH-L1 and GFAP, and the timing of any event wherein said subject may have sustained an injury to the head.

Clause 88. The method of any one of clauses 47 to 87, wherein the sample is a whole blood sample.

Clause 89. The method of any one of clauses 47 to 87, wherein the sample is a serum sample.

Clause 90. The method of any one of clauses 47 to 87, wherein the sample is a plasma sample.

Clause 91. The method of any one of clauses 88-90, wherein the assay is an immunoassay.

Clause 92. The method of any one of clauses 88-90, wherein the assay is a clinical chemistry assay.

Clause 93. The method of any one of clauses 88-90, wherein the assay is a single molecule detection assay.

CLAIMS

What is claimed is:

1. A method of evaluating a human subject for a head injury, the method comprising:

a) performing an assay on a sample obtained from the subject within about 2 hours after a suspected injury to the head to measure a level of an early biomarker in the sample, said early biomarker comprising ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), glial fibrillary acidic protein (GFAP), or a combination thereof; and

b) determining whether the subject has sustained a mild, severe, or a moderate to severe traumatic brain injury (TBI), wherein the subject is determined as having (1) a moderate, severe, or a moderate to severe traumatic brain injury when the level of the early biomarker in the sample is higher than a reference level of the early biomarker or (2) a mild traumatic brain injury when the level of the early biomarker in the sample is lower than a reference level of the early biomarker.

2. The method of claim 1, wherein the subject has received a Glasgow Coma Scale score before or after the assay is performed.

3. The method of claim 2, wherein the subject is suspected as having a moderate, severe, or moderate to severe traumatic brain injury based on the Glasgow Coma Scale score.

4. The method of claim 3, wherein the reference level is correlated with subjects having moderate to severe traumatic brain injury.

5. The method of claim 4, wherein the reference level is correlated with a Glasgow Coma Scale score of 3-12.

6. The method of claim 2, wherein the subject is suspected as having mild traumatic brain injury based on the Glasgow Coma Scale score.

7. The method of claim 6, wherein the reference level is correlated with subjects having mild traumatic brain injury.

8. The method of claim 7, wherein the reference level is correlated with a Glasgow Coma Scale score of 13-15.

9. The method of claim 1, wherein the reference level is correlated with control subjects that have not sustained a head injury.

10. The method of claim 9, wherein the reference level for GFAP is about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL.

11. The method of claim 10, wherein the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL.

12. The method of claim 9, wherein the reference level for UCH-L1 is about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL.

13. The method of claim 12, wherein the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL.

14. The method of any one of claims 1 to 9, wherein the reference level is (a) determined by an assay having a sensitivity of between at least about 65% to 100% and a specificity of between at least about 30% to 100%; (b) determined by an assay having a sensitivity of at least about 99% and a specificity of at least about 75%; (c) between at least about 5 pg/mL to about 3500 pg/mL; or (d) between at least about 10 pg/mL to about 1000 pg/mL.

15. The method of any one of claims 1 to 9 and 14, wherein the reference level for UCH-L1 is from at least about 70 pg/mL to about 3500 pg/mL and the reference level for GFAP is from at least about 5 pg/mL to about 2000 pg/mL.

16. The method of any one of claims 1 to 15, wherein the sample is taken within about 5 minutes, within about 10 minutes, within about 12 minutes, within about 15 minutes, within about 20 minutes, within about 30 minutes, within about 60 minutes, or within about 90 minutes after a suspected injury to the head.

17. The method of any one of claims 1 to 16, further comprising treating the subject assessed as having moderate to severe traumatic brain injury with a traumatic brain injury treatment.

18. The method of any one of claims 1 to 16, further comprising monitoring the subject assessed as having mild traumatic brain injury.

19. A method of evaluating whether to perform a head computerized tomography (CT) scan on a human subject that has sustained or may have sustained a suspected injury to the head, the method comprising:

a) performing an assay on a sample obtained from the subject within about 2 hours after a suspected injury to the head to measure a level of an early biomarker in the sample, said early biomarker comprising ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), glial fibrillary acidic protein (GFAP), or a combination thereof, in the sample; and

b) performing a CT scan on the subject when the level of the early biomarker in the sample is higher than a reference level of the early biomarker and not performing a CT scan on the subject when the level of the early biomarker in the sample is lower than a reference level of the early biomarker.

20. The method of claim 19, wherein the subject has received a CT scan before or after the assay is performed.

21. The method of claim 20, wherein the subject is suspected of having a traumatic brain injury based on the CT scan.

22. The method of any one of claims 19 to 21, wherein the reference level is correlated with positive head computed tomography.

23. The method of claim 19, wherein the reference level is correlated with control subjects that have not sustained a head injury.

24. The method of claim 23, wherein the reference level for GFAP is about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL.

25. The method of claim 24, wherein the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 9.0 pg/mL, about 11.7 pg/mL or about 42.0 pg/mL.

26. The method of claim 25, wherein the reference level for UCH-L1 is about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL.

27. The method of claim 26, wherein the reference level is 1.5 fold, 2.0 fold, 2.5 fold, 3.0 fold, 3.5 fold, 4.0 fold, 4.5 fold or 5.0 fold greater than about 73.5 pg/mL, about 88.2 pg/mL or about 371 pg/mL.

28. The method of any one of claims 19 to 27, wherein the reference level is (a) determined by an assay having a sensitivity of between at least about 65% to 100% and a specificity of between at least about 30% to 100%; (b) determined by an assay having a sensitivity of at least about 99% and a specificity of at least about 75%; (c) between at least about 5 pg/mL to about 3500 pg/mL; or (d) between at least about 10 pg/mL to about 1000 pg/mL.

29. The method of any one of claims 19 to 28, wherein the reference level for UCH-L1 is between at least about 70 pg/mL to about 3500 pg/mL and the reference level for GFAP is between at least about 5 pg/mL to about 2000 pg/mL.

30. The method of any one of claims 19 to 29, wherein the sample is taken within about 5 minutes, within about 10 minutes, within about 12 minutes, within about 15 minutes, within about 20 minutes, within about 30 minutes, within about 60 minutes, or within about 90 minutes after a suspected injury to the head.

31. The method of any one of claims 1 to 30, wherein measuring the level of UCH-L1 is done by an immunoassay or clinical chemistry assay.

32. The method of any one of claims 1 to 31, wherein measuring the level of UCH-L1 comprises:

A. contacting the sample, either simultaneously or sequentially, in any order with:
(1) a UCH-L1-capture antibody, which binds to an epitope on UCH-L1 or UCH-L1 fragment to form a UCH-L1-capture antibody-UCH-L1 antigen complex, and
(2) a UCH-L1-detection antibody which includes a detectable label and binds to an epitope on UCH-L1 that is not bound by the UCH-L1-capture antibody, to form a UCH-L1 antigen-UCH-L1-detection antibody complex,
such that a UCH-L1-capture antibody-UCH-L1 antigen-UCH-L1-detection antibody complex is formed, and

B. measuring the amount or concentration of UCH-L1 in the sample based on the signal generated by the detectable label in the UCH-L1-capture antibody-UCH-L1 antigen-UCH-L1-detection antibody complex.

33. The method of any one of claims 1 to 32, wherein measuring the level of GFAP is done by an immunoassay or clinical chemistry assay.

34. The method of any one of claims 1 to 33, wherein measuring the level of GFAP comprises:

A. contacting the sample, either simultaneously or sequentially, in any order with:

(1) a GFAP-capture antibody, which binds to an epitope on GFAP or GFAP fragment to form a GFAP-capture antibody-GFAP antigen complex, and

(2) a GFAP-detection antibody which includes a detectable label and binds to an epitope on GFAP that is not bound by the GFAP-capture antibody, to form a GFAP antigen-GFAP-detection antibody complex,

such that a GFAP-capture antibody-GFAP antigen-GFAP-detection antibody complex is formed, and

B. measuring the amount or concentration of GFAP in the sample based on the signal generated by the detectable label in the GFAP-capture antibody-GFAP antigen-GFAP-detection antibody complex.

35. The method of any one of claims 1 to 34, wherein the sample is selected from the group consisting of a whole blood sample, a serum sample, a cerebrospinal fluid sample, and a plasma sample.

36. The method of any one of claims 1 to 35, wherein the sample is obtained after the subject sustained an injury to the head caused by physical shaking, blunt impact by an external mechanical or other force that results in a closed or open head trauma, one or more falls, explosions or blasts or other types of blunt force trauma.

37. The method of any one of claims 1 to 35, wherein the sample is obtained after the subject has ingested or been exposed to a chemical, toxin or combination of a chemical and toxin.

38. The method of claim 37, wherein the chemical or toxin is fire, mold, asbestos, a pesticide, an insecticide, an organic solvent, a paint, a glue, a gas, an organic metal, a drug of abuse or one or more combinations thereof.

39. The method of any one of claims 1 to 35, wherein the sample is obtained from a subject that suffers from an autoimmune disease, a metabolic disorder, a brain tumor, hypoxia, a virus, meningitis, hydrocephalus or combinations thereof.

40. The method of any one of claims 1 to 39, wherein said method can be carried out on any subject without regard to factors selected from the group consisting of the subject's clinical condition, the subject's laboratory values, the subject's classification as suffering from mild, moderate, severe, or moderate to severe traumatic brain injury, the subject's exhibition of low or high levels of UCH-L1, GFAP or UCH-L1 and GFAP, and the timing of any event wherein said subject may have sustained an injury to the head.

41. The method of any one of claims 1 to 40, wherein the sample is a whole blood sample.

42. The method of any one of claims 1 to 40, wherein the sample is a serum sample.

43. The method of any one of claims 1 to 40, wherein the sample is a plasma sample.

44. The method of any one of claims 41-43, wherein the assay is an immunoassay.

45. The method of any one of claims 41-43, wherein the assay is a clinical chemistry assay.

46. The method of any one of claims 41-43, wherein the assay is a single molecule detection assay.

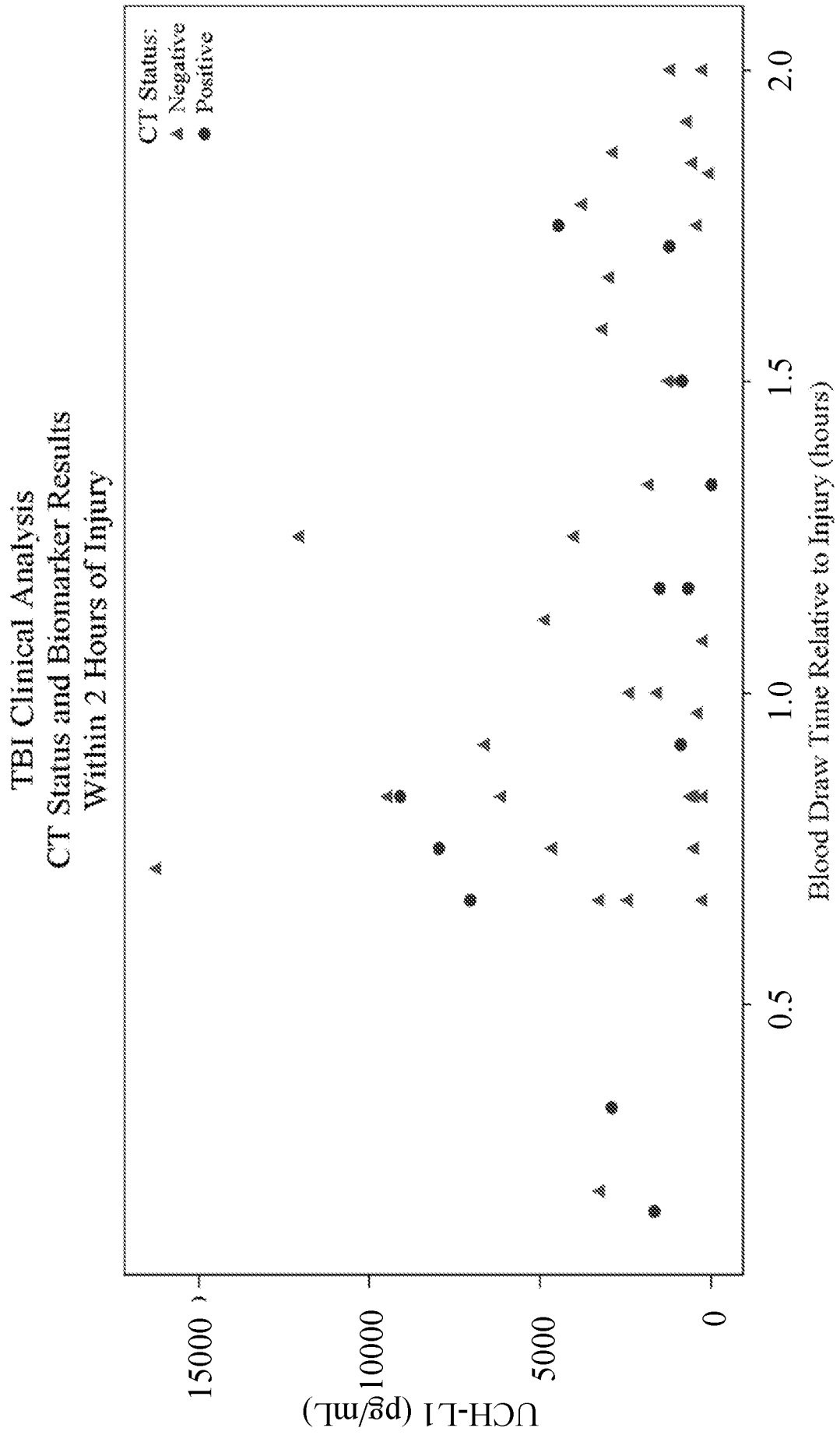
TBI Clinical Analysis
CT Status and Biomarker Results
Within 2 Hours of Injury
CT Status:
Negative
Positive
UCH-L1 (pg/mL)
15000
10000
5000
0
0.5
1.0
1.5
2.0
Blood Draw Time Relative to Injury (hours)

FIG. 1

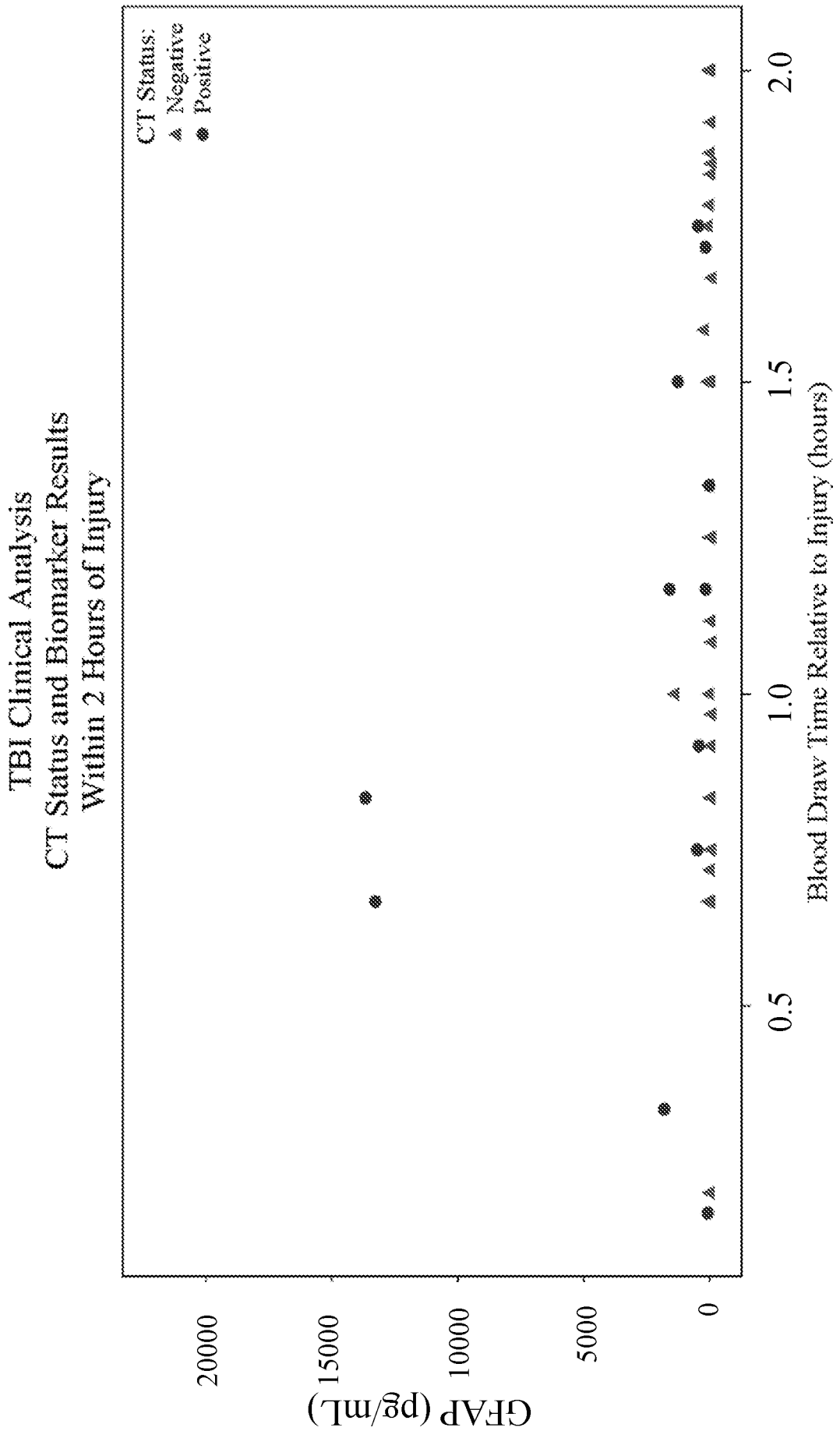
TBI Clinical Analysis
CT Status and Biomarker Results
Within 2 Hours of Injury
CT Status:
Negative
Positive
GFAP (pg/mL)
20000
15000
10000
5000
0
0.5
1.0
1.5
2.0
Blood Draw Time Relative to Injury (hours)

FIG. 2

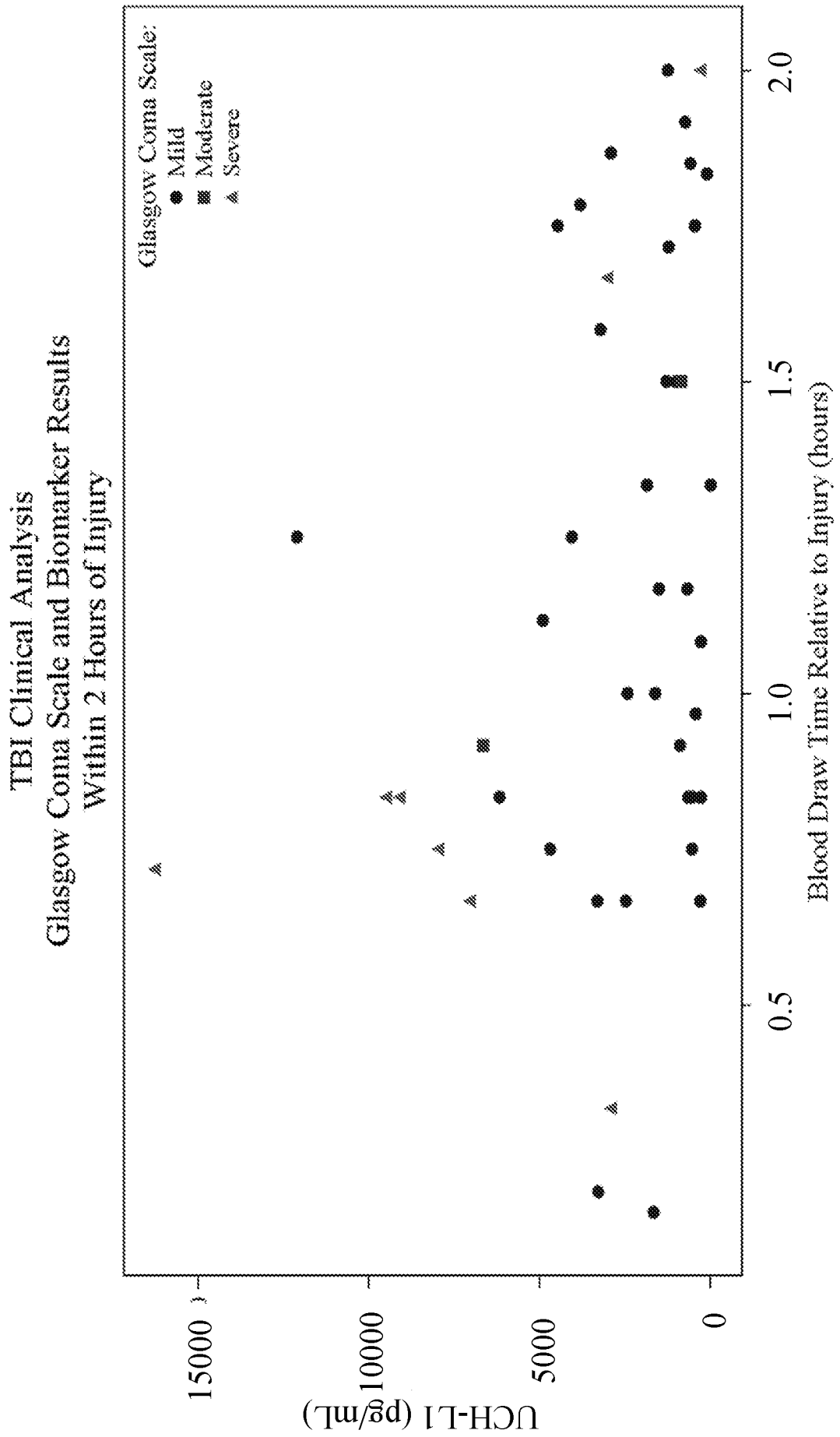
TBI Clinical Analysis
Glasgow Coma Scale and Biomarker Results
Within 2 Hours of Injury
Glasgow Coma Scale:
Mild
Moderate
Severe
UCH-L1 (pg/mL)
15000
10000
5000
0
0.5
1.0
1.5
2.0
Blood Draw Time Relative to Injury (hours)

FIG. 3

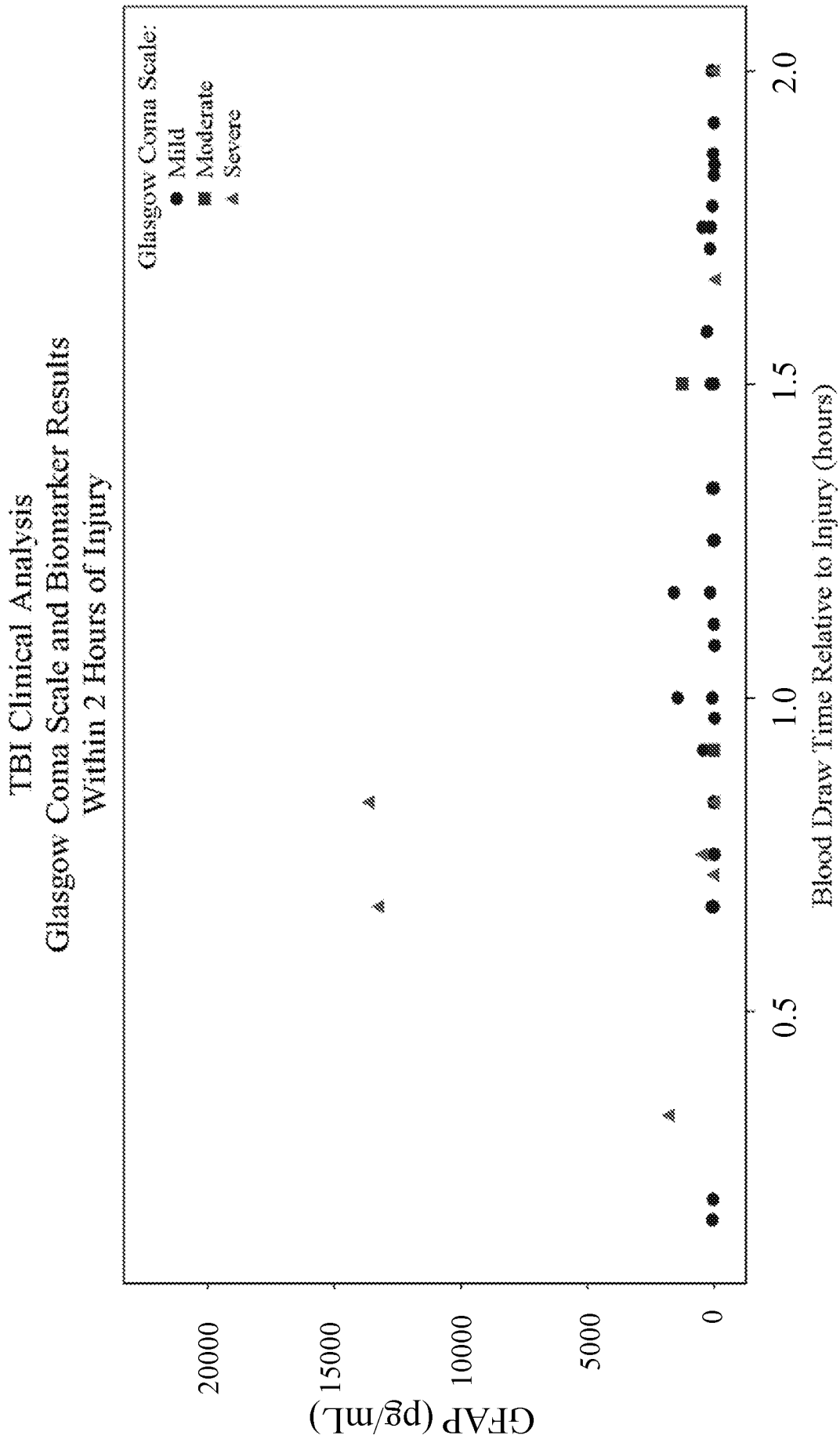
TBI Clinical Analysis
Glasgow Coma Scale and Biomarker Results
Within 2 Hours of Injury
Glasgow Coma Scale:
Mild
Moderate
Severe
GFAP (pg/mL)
20000
15000
10000
5000
0
0.5
1.0
1.5
2.0
Blood Draw Time Relative to Injury (hours)

FIG. 4

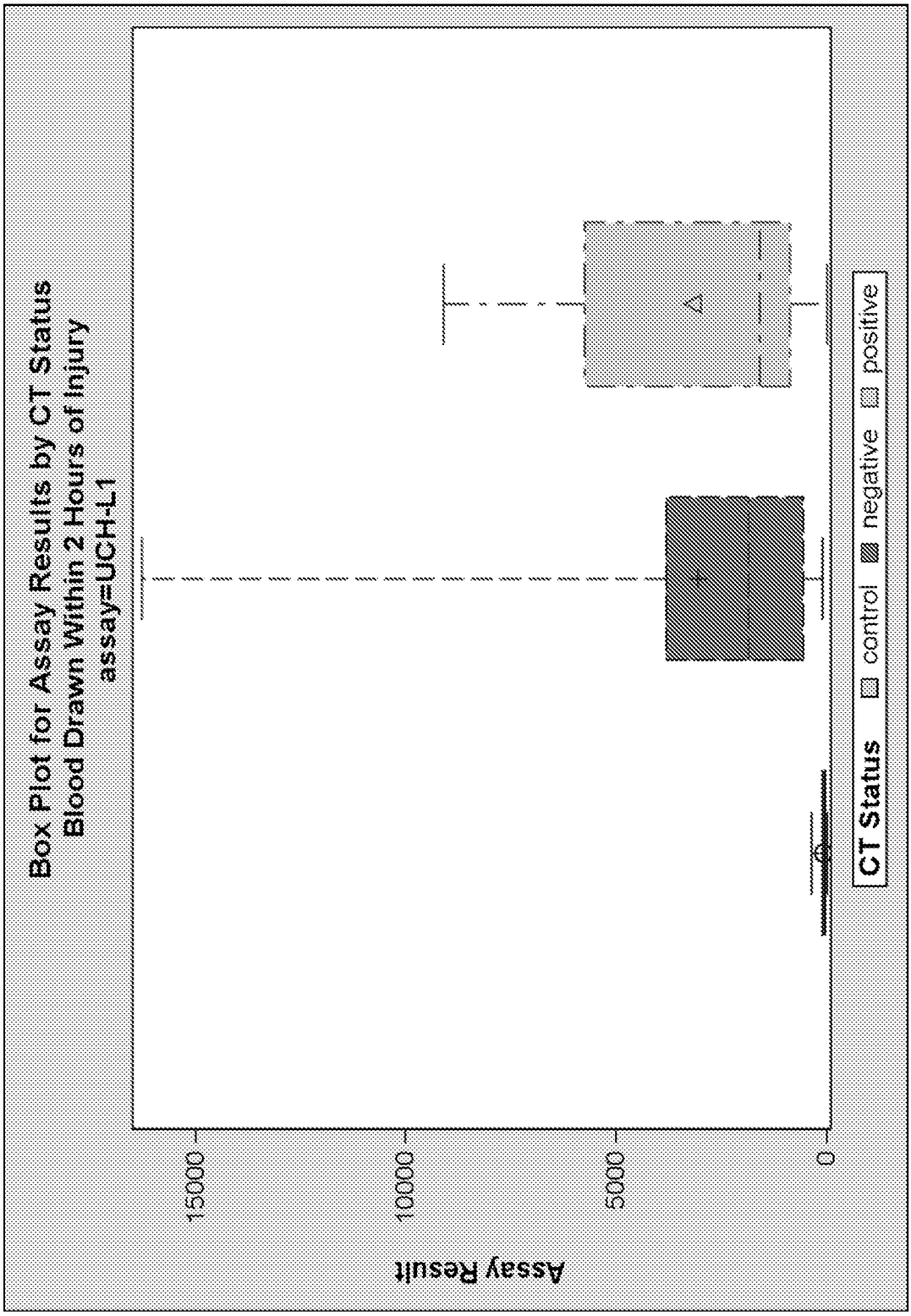
Box Plot for Assay Results by CT Status
Blood Drawn Within 2 Hours of Injury
assay=UCH-L1
Assay Result
15000
10000
5000
0
CT Status
control
negative
positive

FIG. 5

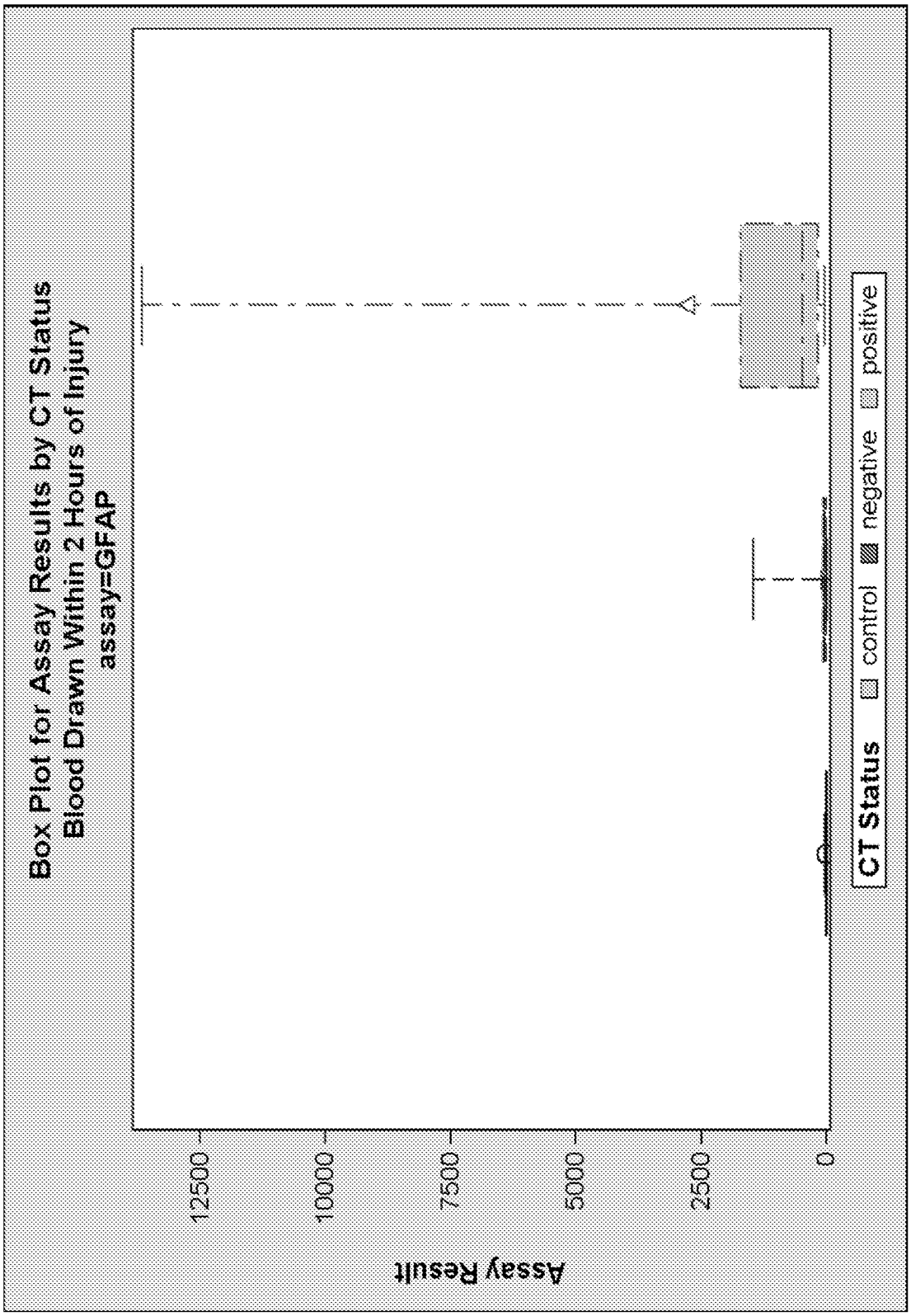
Box Plot for Assay Results by CT Status
Blood Drawn Within 2 Hours of Injury
assay=GFAP
Assay Result
12500
10000
7500
5000
2500
0
CT Status
control
negative
positive

FIG. 6

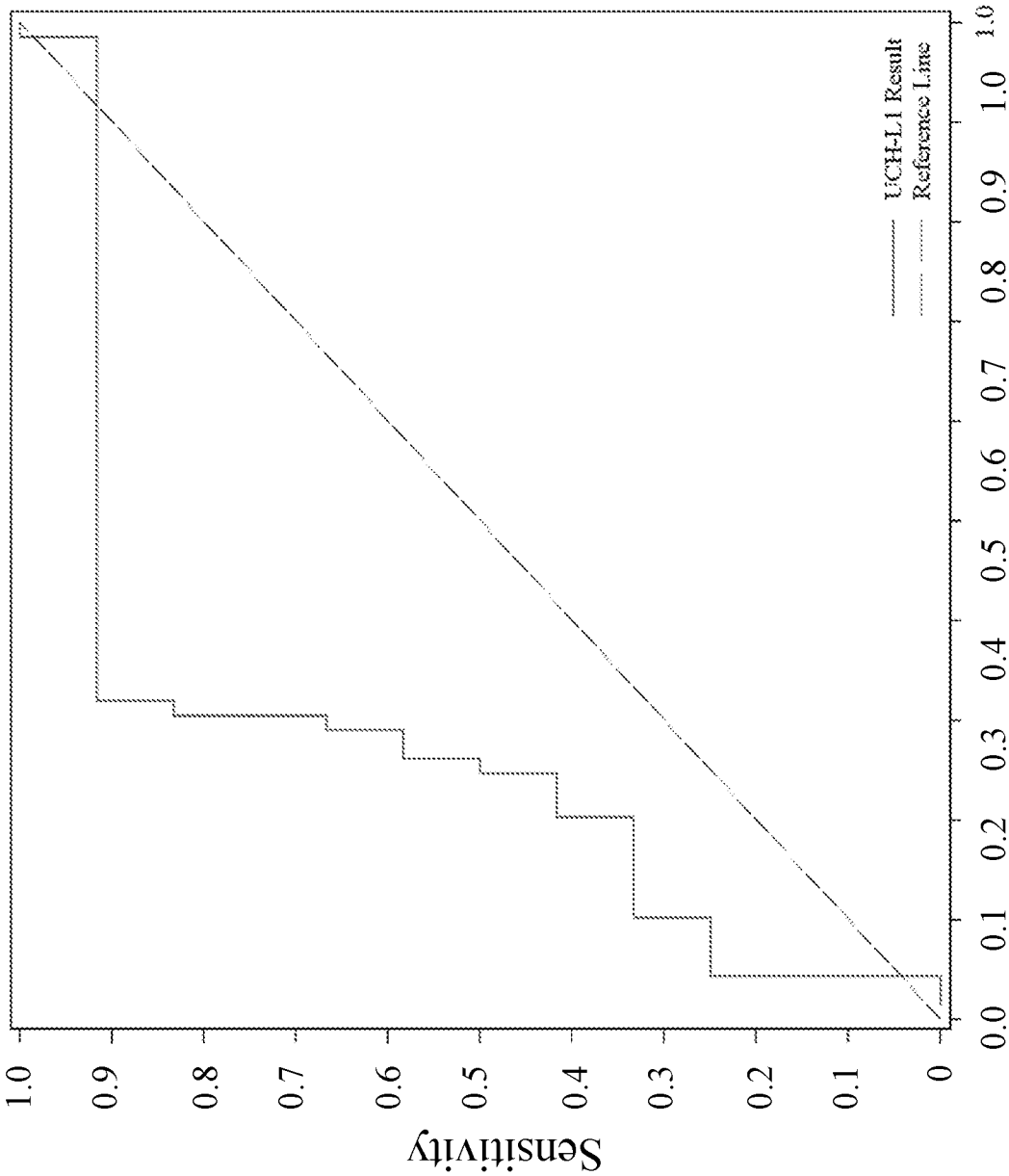
Sensitivity
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1-Specificity
UCH-L1 Result
Reference Line

FIG. 7

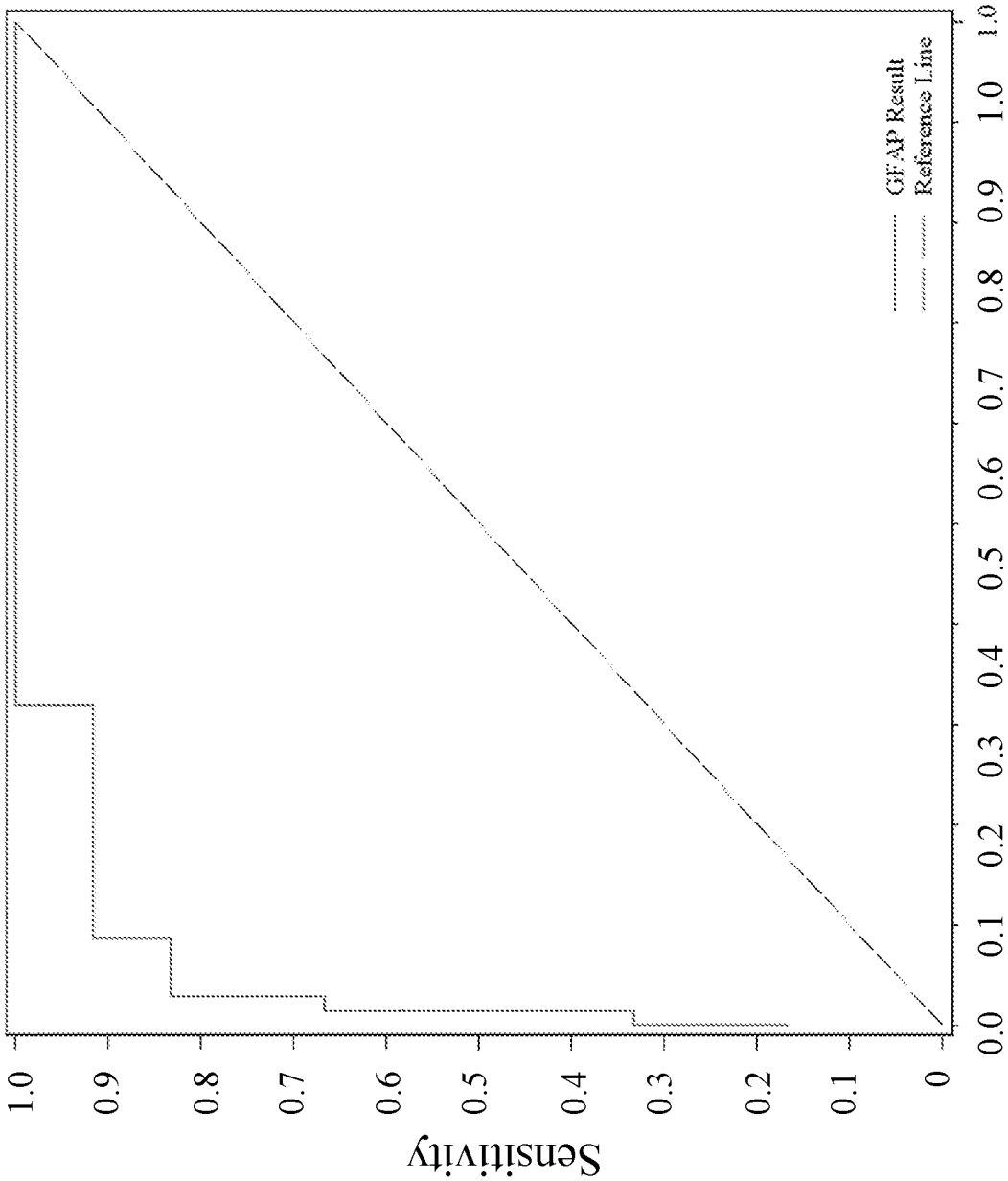
Sensitivity
1-Specificity
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
GFAP Result
Reference Line

FIG. 8

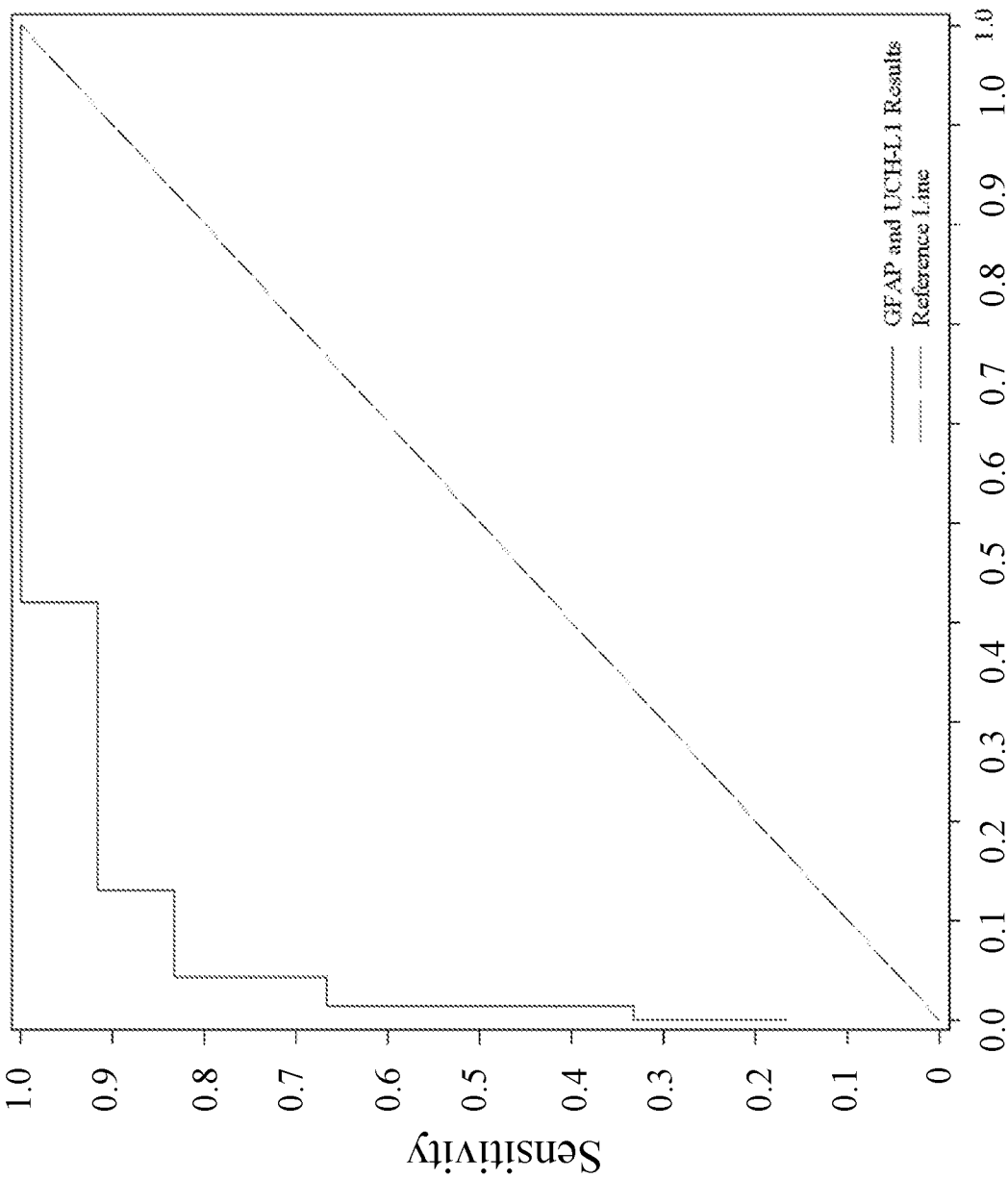
Sensitivity
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1-Specificity
GFAP and UCH-L1 Results
Reference Line

FIG. 9

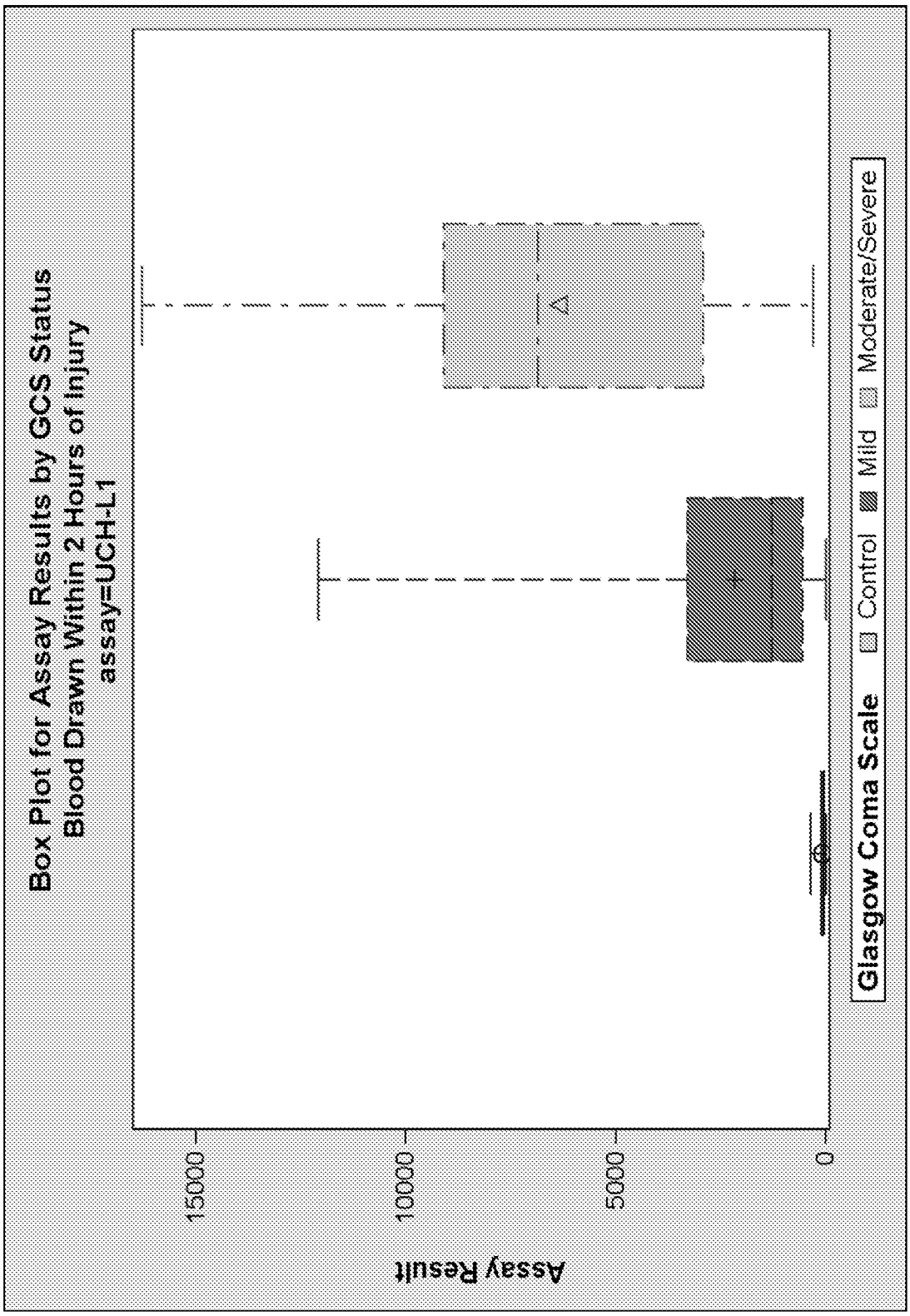
Box Plot for Assay Results by GCS Status
Blood Drawn Within 2 Hours of Injury
assay=UCH-L1
Assay Result
15000
10000
5000
0
Glasgow Coma Scale
Control
Mild
Moderate/Severe

FIG. 10

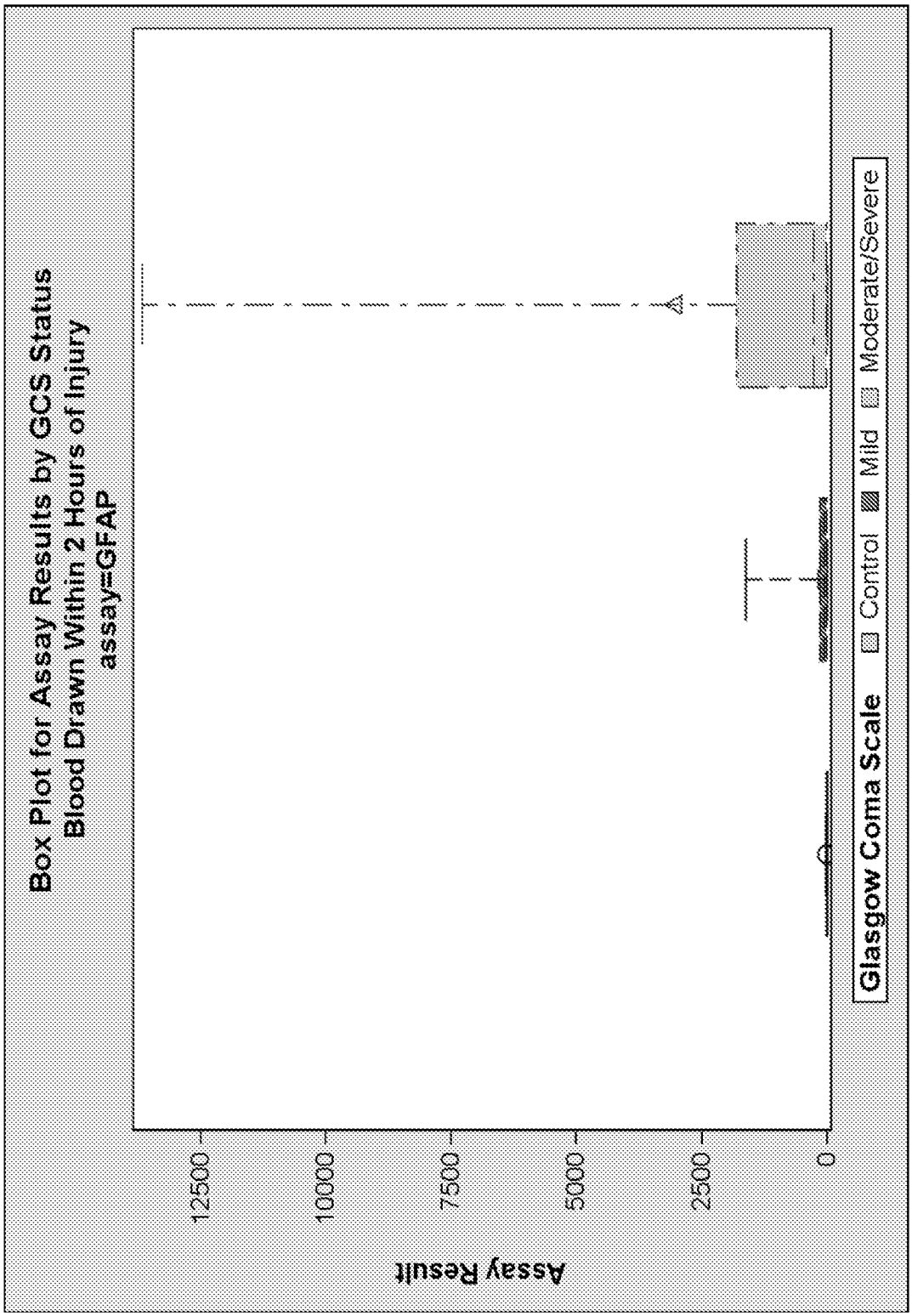
Box Plot for Assay Results by GCS Status
Blood Drawn Within 2 Hours of Injury
assay=GFAP
Assay Result
12500
10000
7500
5000
2500
0
Glasgow Coma Scale
Control
Mild
Moderate/Severe

FIG. 11

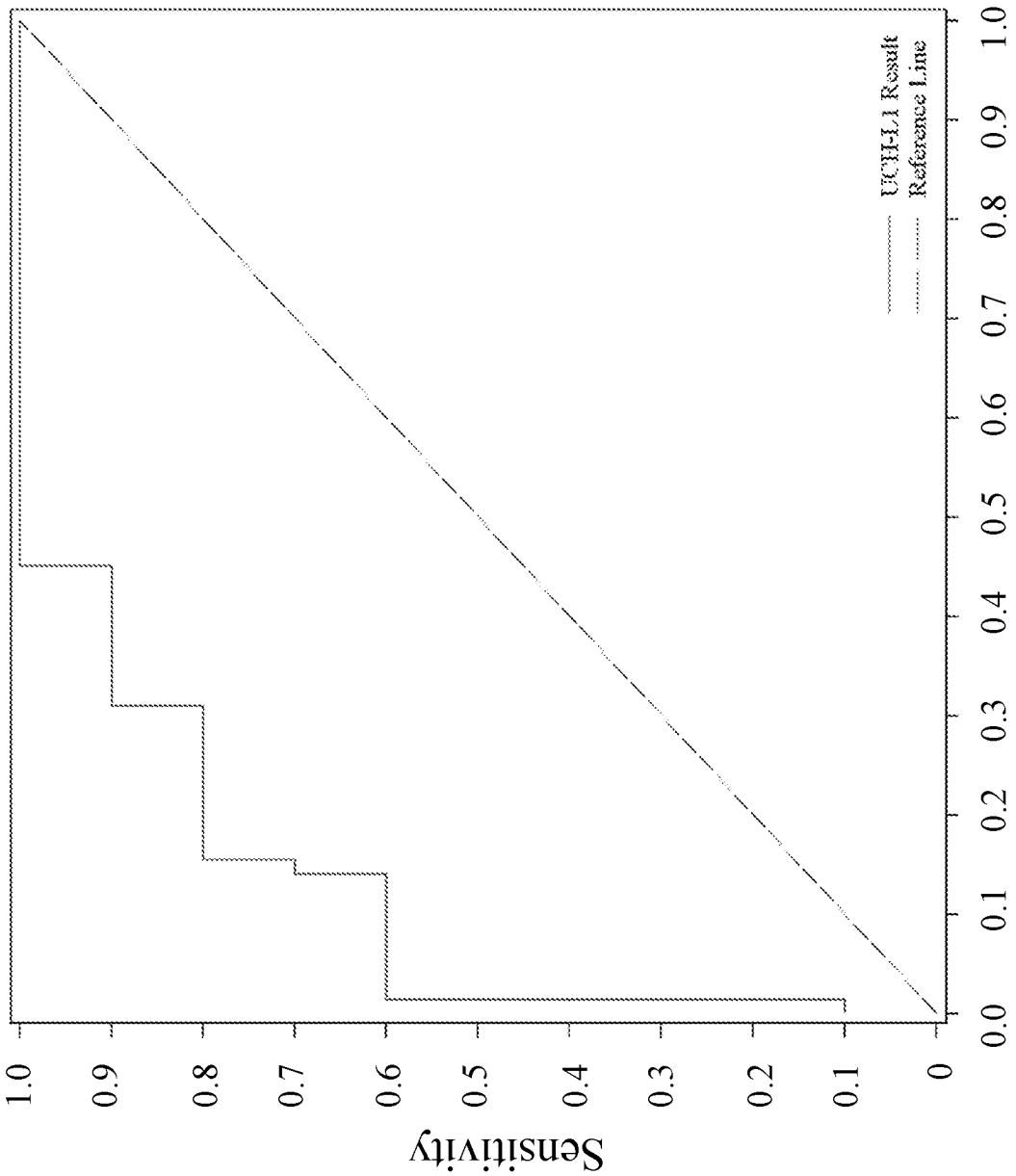
UCHL1 Result
Reference Line
Sensitivity
1-Specificity
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0

FIG. 12

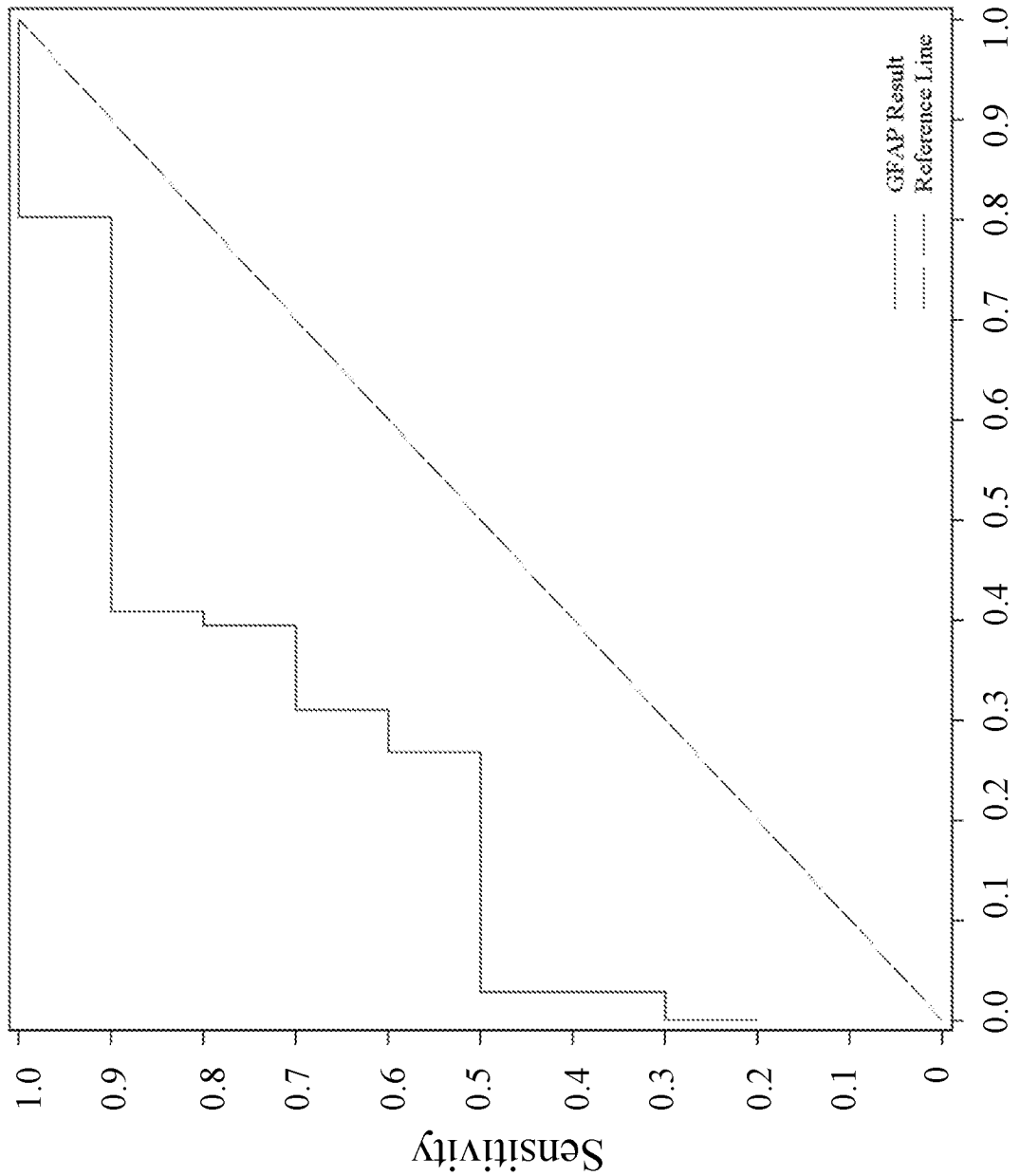
Sensitivity
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1-Specificity
GFAP Result
Reference Line

FIG. 13

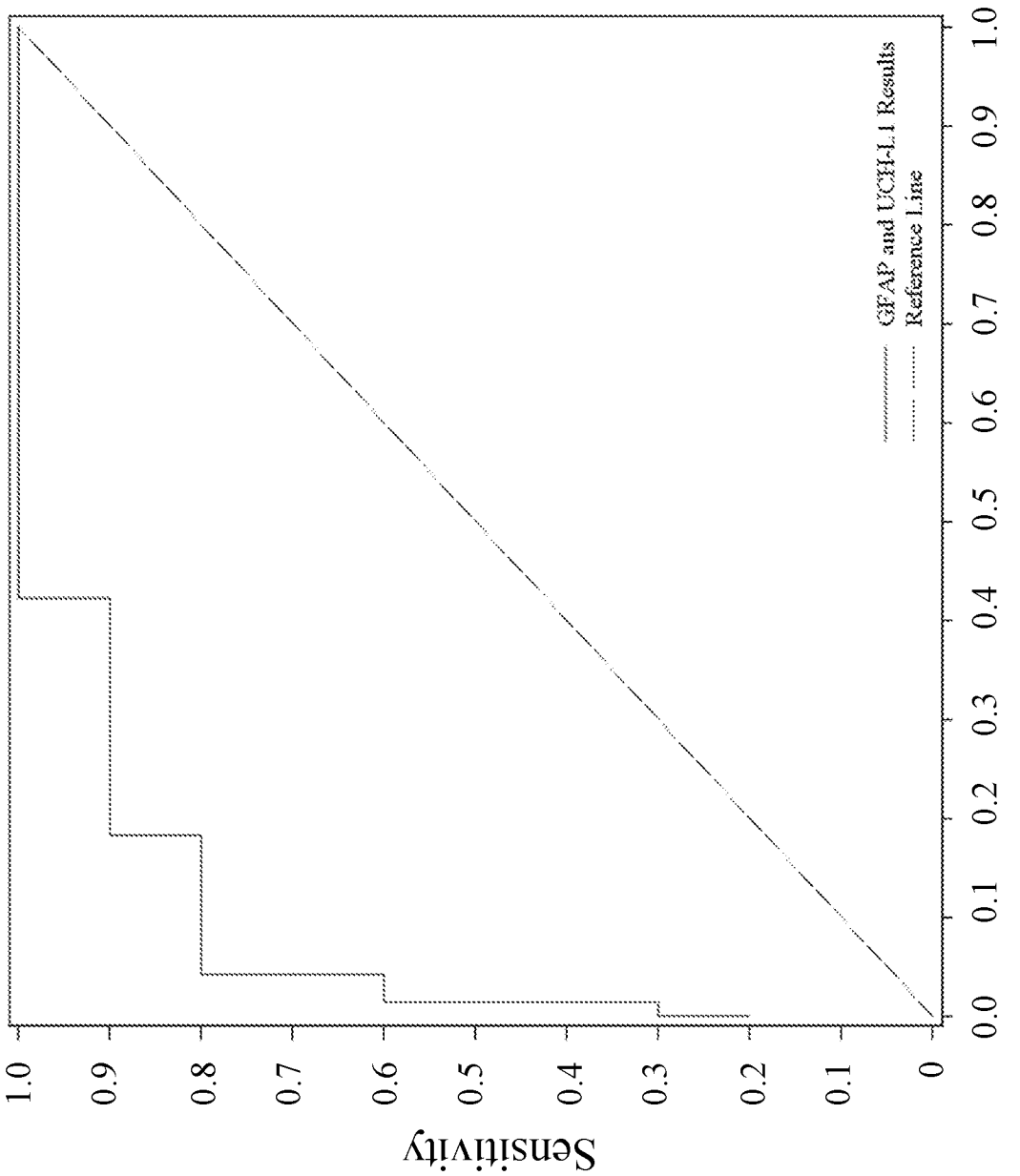
Sensitivity
1-Specificity
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
GFAP and UCH-L1 Results
Reference Line

FIG. 14

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/027353

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/148391 A2 (BANYAN BIOMARKERS, INC. [US]) 23 December 2010 (2010-12-23) abstract example 3 claims 1-27	1-46

X	WO 2015/066211 A1 (BANYAN BIOMARKERS, INC. [US]) 7 May 2015 (2015-05-07) abstract example 9 claims 1-25	1-46

	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 July 2018	02/08/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Giry, Murielle

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/027353

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MONDELLO S. ET AL.: "Serum concentrations of ubiquitin C-terminal hydrolase-L1 and glial fibrillary acidic protein after pediatric traumatic brain injury", SCI. REP., vol. 6, 28203, 20 June 2016 (2016-06-20), pages 1-8, XP055493324, abstract page 2, last paragraph page 3, last paragraph page 4, paragraph 2 page 5, paragraph 2-5	1-46
E	WO 2018/067468 A1 (ABBOTT LABORATORIES [US]) 12 April 2018 (2018-04-12) the whole document	1-46
E	WO 2018/067474 A1 (ABBOTT LABORATORIES [US]) 12 April 2018 (2018-04-12) the whole document	1-46

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/027353

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
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