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(54) Title: METHODS AND COMPOSITIONS FOR DIAGNOSING BRAIN INJURY OR NEURODEGENERATION

(57) Abstract: Methods and compositions for diagnosing brain injury, neurodegeneration; or a predisposition thereto, in a subject are provided. Particularly, the present invention relates to specific antigen antibody reactivities useful in diagnosing brain injury, neurodegeneration or a predisposition thereto, in a subject.

METHODS AND COMPOSITIONS FOR DIAGNOSING BRAIN INJURY OR NEURODEGENERATION

FIELD OF THE INVENTION

The present invention relates to methods and compositions for diagnosing brain injury, neurodegeneration; or a predisposition thereto, and more specifically to clinical methods for determining the presence and type of brain injury or neurodegeneration.

BACKGROUND OF THE INVENTION

Brain injuries are complex and can have multiple severe clinical outcomes. Injury of the brain and spinal cord can result from head trauma, stroke, traumatic birth, heart surgery, cardiac arrest and patients requiring cardiovascular support with ventricular assist devices or extracorporeal membrane oxygenation (ECMO).

About 1.7 Million Americans sustain a traumatic brain injury (TBI) each year, ranging from mild to severe, and this is in addition to about 360,000 soldiers involved in combat operations and public safety workers surviving terrorist attacks who develop mild TBI secondary to explosive (concussive) blasts. It contributes about 30% of all injury related deaths and costs about $60B per year. At least 230,000 people are hospitalized due to TBI and survive; more than a million are treated in an emergency department (ED) for TBI and 80,000 to 90,000 Americans experience long-term disability from TBIs.

Recent study was conducted to determine the dimensions of TBI evaluation in US emergency department. TBI was evaluated during 4.8 million visits per year; and head CT scan was performed in 82% of TBI evaluations (3.9 million visits per year). TBI was diagnosed in 52% of evaluations (2.5 million visits per year). Among those who received head CT scans, 9% had CT evidence of traumatic abnormalities. Among patients evaluated for TBI who had a Glasgow Coma Scale score recorded, 94.5% were classified as having mild TBI, 2.1% as moderate TBI, and 3.5% as severe TBI. Among patients with International Classification of Diseases, Ninth Revision, Clinical Modification, codes permitting the calculation of head Abbreviated Injury Scale scores 9.0%, 85.0%, 2.5%, 3.2%, 0.3%, and 0% had head Abbreviated Injury Scale scores of 1, 2, 3, 4, 5, and 6, respectively. Of patients evaluated for TBI, 31% had other head/face/neck injuries, 10%

had spine and back injuries, 7% had torso injuries, and 14% had extremity injuries (Korley et al., Sep 10, 2015, J Head Trauma Rehabil)

TBI is the result of a blunt blow, jolt or blast overpressure to the head that disrupts brain function. The subset of mild TBI (mTBI) has represented a harder segment of TBI to diagnose. The severity of head injuries range from a brief change in mental status or consciousness to extended unconsciousness and amnesia. In severe or multiple concussion cases, personality changes can occur with devastating results. Cognitive decline is recognized as part of the post injury syndrome.

Proper treatment of TBI injury requires an accurate diagnosis of the structures affected. The mechanisms of injury in TBI cause a variety of abnormalities in the peripheral vestibular mechanisms, central vestibular structures, ocular-motor tracts, cerebellum, as well as all portions of the brain communicating with these structures. The onset of vestibular deficits generally occurs within seven to ten days post injury. While reported symptoms of dizziness resolve after three months, 15% have persistent symptoms one year later.

At present, one of the rather subjective and not totally effective diagnostic procedures when traumatic brain injury is suspected involves a number of examining techniques. The patient receives a neurological examination which may consist of the following: 1) mental status, 2) motor function, 3) sensory examination, 4) deep tendon reflexes, 5) station, gait, and equilibrium, and 6) cranial nerve function. The mental status examination may include: a) level of consciousness, b) short and long term memory, c) knowledge of patient and place and d) questions about symptoms: headache, dizziness, blurry vision, etc. In addition, the patient may also have radiological studies which could include CT scan of the head, MRI, PET scan. It has been reported that in the early stages of (especially mild) traumatic brain injury, the imaging techniques may not be sufficiently sensitive to detect an abnormality. Furthermore, the patient's cognitive skills may not be impaired initially, and there may be few, if any, symptoms. Patients are often observed over 24-48 hours and are awakened at regular intervals (e.g., every 3-4 hours) to assure that they are able to be aroused. Narcotics for headache or other pain are not given, so that their effects do not cloud the issue of the patient's arousal state. A computerized test which determines level of cognition and reaction time is often employed with repetitive examinations.

One of the problems with this approach in diagnosing potential traumatic brain injuries is that it is not one which always provides precise, timely, objective information. It is also subject to individual variations from person-to-person. Further, if the person is asymptomatic at the time, the conclusion might be that there is no problem, and the individual might be encouraged to go back to normal activities. Such guidance could potentially be injurious to the person's health and could even lead to fatal consequences.

Once a patient has been diagnosed with a brain injury, it becomes important to treat the patient in a timely, effective manner in order to minimize the risk of permanent injury or death.

In spite of the foregoing known procedures, there remains a very real and substantial need for a method of early and effective determination as to whether an individual has suffered a brain injury, how severe it might be, and upon finding the presence of such an injury, effectively treating the patient.

SUMMARY OF THE INVENTION

The present invention provides methods and kits for diagnosing brain injury or neurodegeneration. The present invention further provides antigen probe arrays for practicing such a diagnosis, and antigen probe sets for generating such arrays.

The antigen probe sets of the present invention can be used to profile the antibody response to said antigens in patients suffering from brain injury or neurodegenerative disease, due to disruption of any or all components of the anatomic structure and the ability to detect elements which cross the blood brain barrier. This antibody response profile can be used for the diagnosis, monitoring and management of brain injury. According to some embodiments, the antibody profile reflects the patient status at time of injury. The profile of the antibody response can be measured on any platform including but not limited to a micro array or any array chip.

The present invention is based, in part, on the unexpected results obtained when testing the antibody reactivity of patients suffering from brain injury compared to healthy controls. Surprisingly, differential immunoglobulin G (IgG) and IgM reactivities to specific antigens were found in the tested brain injury patients, compared to healthy controls. The

present invention is also based on the discovery that analysis of the pattern of an individual's antibody response to specific brain related molecules in combination with markers of immune response provides a novel and reliable method of ascertaining the nature and extent of brain injury and of other neurodegenerative conditions. Thus, the present invention provides unique antigens, indicative to brain injury. The present invention further provides antigen-autoantibody reactivity patterns relevant to brain injury. In particular embodiments, the present invention provides highly specific, reliable, accurate and discriminatory assays for diagnosing and monitoring brain injury, based on the indicative antigens, or on reactivity patterns thereof.

According to some embodiments, the 'pre-existing state' of the patient status at time of injury is monitored.

Thus, according to some embodiments of the invention, there are provided novel methods for diagnosing and monitoring the progression of brain injury. According to some embodiments of the invention, the methods comprise determining the reactivity of antibodies in a sample obtained or derived from a subject to at least one antigen as described herein. The methods of the invention further comprise a step of comparing the reactivity of antibodies in the sample to the at least one antigen to a control reactivity to said at least one antigen. According to certain embodiments, a significantly differential reactivity of the antibodies in the sample compared to the reactivity of the healthy control, or to the reactivity of baseline samples from the same patient, is an indication that the subject is afflicted with brain injury.

According to certain embodiments, the baseline samples from the same patient may be used [for measurements over time] to predict progression, resolution of event or remission of disease course.

According to certain embodiments, the methods of the present invention can discriminate which patients with brain injury require a head CT scan to rule out intracranial hemorrhage versus concussion alone. If implemented as an initial response (e.g., in the emergency department (ED) setting) or later (e.g., neurology department), the methods of present invention would decrease head CT scan utilization, decreasing health care costs and radiation exposure.

Thus, according to a first aspect, the present invention provides a method of diagnosing brain injury in a subject, the method comprising the steps of obtaining a sample from the subject, determining the reactivity of antibodies in the sample to at least one antigen selected from the groups consisting of SEQ ID NOs:1-115, isoforms thereof, post-translationally modified forms thereof, fragments thereof, or combinations of any of the foregoing; and comparing the reactivity of antibodies in the sample to a reactivity of a healthy control; wherein a significantly different reactivity of the antibody or antibodies in the sample compared to the reactivity of the healthy control is an indication that the subject is afflicted with brain injury.

In certain embodiments, the at least one antigen is selected from the groups consisting of SEQ ID NOs: 2, 14, 28, 42, 85, and 86, or any combination thereof.

In certain embodiments, said method further comprising measuring the levels of one or more biomarkers in the sample; and comparing the levels of the one or more biomarkers with predefined levels of the same biomarkers that correlate to a subject having brain injury and predefined levels of the same biomarkers that correlate to a healthy control, wherein a correlation to one of the predefined levels provides the diagnosis.

In certain embodiments, said brain injury is selected from the group consisting of: concussions, chronic traumatic encephalopathy, mild traumatic brain injuries, moderate traumatic brain injuries, severe traumatic brain injuries, head trauma, concussive blasts and brain neurodegenerative condition.

In certain embodiments, said brain injury causes disruption of the blood-brain barrier.

In certain embodiments, the brain neurodegenerative condition further comprises loss of memory or motor function and cognitive decline.

In certain embodiments, the neurodegenerative condition is selected from the group consisting of: Alzheimer's disease, Huntington's disease, Parkinson's disease, demyelinating disease, HTLV-1-associated myelopathy (HAM), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), pathological neurological symptoms after injury or trauma, encephalopathy and viral encephalopathy.

In certain embodiments, a significantly higher reactivity of the antibodies in the sample compared to the reactivity of the healthy control is an indication that the subject is of increased likelihood to be afflicted with brain injury. In other certain embodiments, where the reactivity of the antibodies in the sample compared to the reactivity of the healthy control is not significantly higher, where the reactivity of the antibodies in the sample compared to the reactivity of the healthy control is the same, where the reactivity of the antibodies in the sample compared to the reactivity of the healthy control is lower or where the reactivity of the antibodies in the sample compared to the reactivity of the healthy control is significantly lower, it is an indication that the subject is of decreased likelihood to be afflicted with brain injury. Each possibility represents a separate embodiment of the present invention.

In certain embodiments of the methods of the present invention, the methods are preceded by a step comprising obtaining or deriving a sample from the subject. In certain embodiments, the sample is obtained or derived from the subject by non-invasive means or methods.

In certain embodiments, said obtaining is carried out within two hours of the head trauma. In certain embodiments, said obtaining is carried out within four hours of the head trauma. In certain embodiments, said obtaining is carried out within 24 hours of the head trauma. In certain embodiments, said obtaining is carried out within 72 hours of the head trauma. In certain embodiments, said obtaining is carried out during the post-acute care.

In certain embodiments, the subject is conscious at the time of said obtaining.

In certain embodiments, determining the reactivity of antibodies in the sample to a plurality of antigens produces a reactivity pattern, used for the diagnosis of brain injury in the subject. Thus, according to exemplary embodiments of the invention, the reactivity pattern of antibodies in the sample to the plurality of antigens is compared to the reactivity pattern of antibodies in a sample corresponding to healthy control subjects to said plurality of antigens, wherein a significant difference between the reactivity pattern of the sample and the reactivity pattern of healthy controls indicates that the subject is afflicted with, or in other embodiments has increased likelihood for having brain injury. Conveniently, the reactivity patterns are calculated and compared using e.g. learning and pattern recognition algorithms as described herein.

According to another embodiment, the reactivity of antibodies comprises IgG and IgM reactivities. According to another embodiment, the significantly higher reactivity of the antibodies in the sample comprises differential IgG and/or IgM reactivities. According to another embodiment, the increased IgM reactivity is of at least one antigen selected from the group consisting of SEQ ID NOs: 1-115, isoforms thereof, post-translationally modified forms thereof, fragments thereof, or combinations of any of the foregoing. According to another embodiment, the increased IgG reactivity is of at least one antigen selected from the group consisting of SEQ ID NOs: 1-115, isoforms thereof, post-translationally modified forms thereof, fragments thereof, or combinations of any of the foregoing. Each possibility represents a separate embodiment of the invention.

According to additional embodiments of the methods of the present invention, the sample obtained from the subject is a biological fluid. According to some embodiments, the sample is selected from the group consisting of plasma, serum, blood, cerebrospinal fluid, synovial fluid, sputum, urine, saliva, tears, lymph specimen, or any other biological fluid known in the art. Each possibility represents a separate embodiment of the invention. According to certain embodiments, the sample obtained from the subject is selected from the group consisting of serum, plasma and blood. According to one embodiment, the sample is a serum sample. In certain embodiments, the sample is obtained or derived from the subject by non-invasive means or methods.

According to certain embodiments of the methods of the present invention, the control is selected from the group consisting of a sample from at least one healthy individual, a baseline sample from same subject, a panel of control samples from a set of healthy individuals, and a stored set of data from healthy individuals. Each possibility represents a separate embodiment of the invention. Typically, a healthy individual is a subject not afflicted with brain injury. In another embodiment, a healthy individual is a subject not afflicted with neurodegenerative disease.

According to another embodiment, the method comprises determining the reactivity of antibodies in the sample to a plurality of antigens.

According to another embodiment, the method comprises determining the reactivity of antibodies in the sample to at least one antigen selected from the group consisting of SEQ ID NOs: 1-115, isoforms thereof, post-translationally modified forms thereof, fragments

thereof, or combinations of any of the foregoing. According to another embodiment, the method comprises determining the reactivity of antibodies in the sample to at least two antigens selected from the group consisting of SEQ ID NOs: 1-115, isoforms thereof, post-translationally modified forms thereof, fragments thereof, or combinations of any of the foregoing.

According to another embodiment, the plurality of antigens is used in the form of an antigen probe set, an antigen array, or an antigen chip.

According to another aspect, the present invention provides an antigen probe set comprising a plurality of antigen probes selected from the group consisting of SEQ ID NOs: 1-115, isoforms thereof, post-translationally modified forms thereof, fragments thereof, or combinations of any of the foregoing. In another embodiment, the antigen probe set comprises the antigen probes of SEQ ID NOs: 1-115, isoforms thereof, post-translationally modified forms thereof, fragments thereof, or combinations of any of the foregoing.

According to another aspect, the present invention provides an article of manufacture comprising the antigen probe set described above.

In certain embodiments, the article of manufacture, further comprising one or more biomarkers selected from the group consisting of glial fibrillary acidic protein (GFAP), Synuclein beta (Sncb), Metallothionein-3 (MT3), Neurogranin (NRGN), intercellular adhesion molecule-5 (ICAM5) and Brain derived neurotrophic factor (BDNF), or citrullinated forms thereof.

In certain embodiments, the article of manufacture is in the form of an antigen probe array or in the form of an antigen chip or in the form of a dipstick or in the form of a lateral flow test or in the form of an ELISA plate or in the form of a Quanterix system, an Agilent Plate reader, a Meso Scale Diagnostics platform, or any other platform known to those skilled in the art. In certain embodiments, the article of manufacture is in the form of a kit.

According to certain embodiments, the kit further comprises means for determining the reactivity of antibodies in a sample to at least one antigen of the plurality of antigens. According to another embodiment, the kit further comprises means for comparing reactivity of antibody in different samples to at least one antigen of the plurality of antigens.

According to another embodiment, the kit further comprises instructions for use of the kit for diagnosing brain injury.

According to another aspect, there is provided use of the at least one antigen selected from the group consisting of: SEQ ID NOs: 1-115, isoforms thereof, post-translationally modified forms thereof, fragments thereof, or combinations of any of the foregoing; for the preparation of a diagnostic kit for diagnosing brain injury in a subject. Each possibility represents a separate embodiment of the invention. The diagnostic kit is, in some embodiments, useful for determining the reactivity of antibodies in a sample, thereby determining the reactivity pattern of the sample to the at least one antigen. In some embodiments, a significant difference (e.g., increase) between the reactivity pattern of the sample compared to a reactivity pattern of a control sample is an indication for brain injury.

According to another aspect, there is provided a method for qualifying brain injury status in a subject the method comprising the steps of: obtaining a sample from the subject; determining the reactivity of antibodies in the sample to at least one antigen selected from the group consisting of SEQ ID NOs: 1-115, isoforms thereof, post-translationally modified forms thereof, fragments thereof, or combinations of any of the foregoing; and comparing the reactivity of antibodies in the sample to a predefined reactivity that correlate to one or more brain injury statuses selected from the group consisting of having brain injury, not having brain injury, predisposition to brain injury, sub-acute brain injury, acute brain injury, post-acute brain injury, progressing brain injury, regressing brain injury, subclinical brain injury, mild brain injury, moderate brain injury, severe brain injury and chronic brain injury, wherein a correlation to one of the predefined reactivities determines the brain injury status of the subject.

In certain embodiments, said method further comprising measuring the levels of one or more biomarkers in the sample; and comparing the levels of the one or more biomarkers with predefined levels of the same biomarkers that correlate to one or more brain injury statuses, wherein a correlation to one of the predefined levels determines the brain injury status of the subject.

According to another aspect, there is provided a method of detecting recovery from brain injury in a subject, the method comprising the steps of: obtaining a sample from the subject; determining the reactivity of antibodies in the sample to at least one antigen selected from the group consisting of SEQ ID NOS: 10, 44, 61, 66, 102, 104, isoforms

thereof, post-translationally modified forms thereof, fragments thereof, or any combinations thereof; and comparing the reactivity of antibodies in the sample to a predefined reactivity threshold; wherein a significantly different reactivity of the antibodies in the sample compared to the predefined reactivity threshold is indicative of recovery from brain injury in said subject.

In certain embodiments, said method further comprising measuring the levels of one or more biomarkers in the sample; and comparing the levels of the one or more biomarkers with predefined levels of the same biomarkers that correlates with recovery from brain injury, wherein a correlation to one of the predefined levels is indicative of recovery from brain injury of the subject.

In certain embodiments, the one or more biomarkers is selected from the group consisting of glial fibrillary acidic protein (GFAP), Synuclein beta (Sncb), Metallothionein-3 (MT3), Neurogranin (NRGN), intercellular adhesion molecule-5 (ICAM5) and Brain derived neurotrophic factor (BDNF), or citrullinated forms thereof.

In certain embodiments, a combination of one or more antibodies and one or more biomarkers are used.

According to certain embodiments, the comparison is conducted by using at least one classifier algorithm.

According to certain embodiments, the at least one classifier algorithm is selected from the group consisting of a decision tree classifier, logistic regression classifier, nearest neighbor classifier, neural network classifier, Gaussian mixture model (GMM), Support Vector Machine (SVM) classifier, nearest centroid classifier, linear regression classifier, linear discriminant analysis (LDA) classifier, quadratic discriminant analysis (QDA) classifier and random forest classifier.

Other objects, features and advantages of the present invention will become clear from the following description and drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates anti-Fatty acid-binding protein (FABP-3, SEQ ID No: 61) IgM autoantibody levels at day 30 post brain injury. TBI patients with Glasgow Outcome Scale

Extended (GOSE) score <8 (cross labeled) represent lower IgM levels than patients with GOSE score=8 (circle labeled).

Figure 2 illustrates anti-Myelin basic protein (MBPR149, SEQ ID No: 10) derived BSA conjugated peptide IgM autoantibody levels at day 30 post brain injury. TBI patients with Glasgow Outcome Scale Extended (GOSE) score <8 (cross labeled) represent higher IgM levels than patients with GOSE score=8 (circle labeled).

Figure 3 illustrates anti-Myeloperoxidase (MPO, SEQ ID No: 85) IgM autoantibody levels in serum samples obtained from TBI patients (circle labeled) in comparison with healthy controls (cross labeled).

Figure 4A illustrates anti-CMV (SEQ ID No: 86) IgG autoantibody levels in serum samples obtained from TBI patients (circle labeled) at day 30 and day 90 post injury (N=142) in comparison with healthy controls (cross labeled) (N=21).

Figure 4B shows the above separation performance by receivers operating characteristic (ROC) curves of anti-Cytomegalovirus (CMV) IgG autoantibody levels. T test P value for separation: 3.746E-07, after FDR correction: 5.02E-05. Kruskal-Wallis test P value for separation: 4.567E-05, after FDR correction: 0.0081593.

Figure 5A demonstrates the prediction of the clinical status of TBI patients at day 90 post injury, based on the anti-TNFRSF12A (SEQ ID No: 104) IgM autoantibody levels in serum samples obtained from TBI patients at day 30 post injury. TBI patients, with GOSE <8 at day 90 post injury (circle labeled) (N=52) were compared with TBI patients, with GOSE =8 at day 90 post injury (cross labeled) (N=15).

Figure 5B shows the above separation performance by receivers operating characteristic (ROC) curves of anti-TNFRSF12A IgM autoantibody levels. T test P value for separation: 6.808E-06, after FDR correction: 0.0036493. Kruskal-Wallis test P value for separation: 0.0004082, after FDR correction: 0.1541973

Figure 6 shows the area under the Receiver Operating Characteristics (ROC) curves of six classification methods (SVM, LR, QDA, CART, RF and LDA) based on 100 iterations of 70:30 cross validation. Features were ranked according to their median scoring or frequency of model inclusion, depending on the method.

Serum samples obtained from TBI patients at time 0 (t0, N=85) were compared with serum samples obtained from healthy control (HC, N=21). The analysis was based on 464 iChip features (232 antigen, IgM and IgG) and four ELISA features. iChip data is based on average of two block replicates, following correction procedure. ELISA features were selected based on data availability; only features with data available for > 80% of the iChip samples were used. Samples with missing ELISA data were removed from the analysis.

Figure 7 shows ROC curves of six classification methods (SVM, LR, QDA, CART, RF and LDA), based on 100 iterations of 70:30 cross validation. Features were ranked according to their median scoring or frequency of model inclusion, depending on the method. Serum samples obtained from TBI patients at time 0 (t0) with abnormal CT were compared with samples obtained from TBI patients at time 0 (t0) with Normal CT. Analysis was based on 464 iChip features (232 antigen, IgM and IgG) and four ELISA features. iChip data is based on average of two block replicates, following correction procedure. ELISA features were selected based on data availability; only features with data available for > 80% of the iChip samples were used. Samples with missing ELISA data were removed from the analysis.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods of diagnosing brain injury or a neurodegenerative disorder in a subject. The present invention further provides antigen probe sets or arrays for practicing such a diagnosis, and identifies specific antigen probe sets for generating such sets or arrays.

Without wishing to be bound by any particular theory or mechanism of action, the invention is based, in part, on the finding of unique antigens highly distinctive between healthy subjects and patients suffering from brain injury. The invention is further based on the finding that the antibody reactivity profile in serum of patients suffering from brain injury was clearly distinct from healthy control individuals. Although protein biomarkers of brain injury patients have been extensively investigated, the unique antibody immune signatures as described herein have not been described before. Advantageously, the unique antibody signatures of the present disclosure provide highly sensitive and specific assays for diagnosing brain injury.

The present invention provides, in some embodiments, unique antigen-antibody reactivity patterns particularly relevant to brain injury. In the course of investigating specific antibodies, the inventors examined the reactivity of IgM and IgG antibodies in the sera of healthy persons and those diagnosed with brain injury to a variety of antigens, using antigen microarray and informatics analysis.

DEFINITIONS

It is to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting. It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise.

The term "about" as used herein means in quantitative terms plus or minus 5%, or in another embodiment plus or minus 10%, or in another embodiment plus or minus 15%, or in another embodiment plus or minus 20%.

As used herein, the term "autoantibodies" refers to antibodies that are capable of reacting against an antigenic constituent of an individual's own tissue or cells (e.g., the antibodies recognize and bind to "self-antigens").

The term "brain injury" refers to a condition in which the brain is damaged by injury caused by an event. As used herein, an "injury" is an alteration in cellular or molecular integrity, activity, level, robustness, state, or other alteration that is traceable to an event. For example, an injury includes a physical, mechanical, chemical, biological, functional, infectious, or other modulator of cellular or molecular characteristics. An event can include a physical trauma such as an impact (percussive or concussive) or a biological abnormality such as a stroke resulting from either blockade or leakage of a blood vessel. An event is optionally an infection by an infectious agent. A person of skill in the art recognizes numerous equivalent events that are encompassed by the terms injury or event.

According to some embodiments of the method of the present invention, a healthy subject's predisposition to future onset of brain injury is also diagnosed. According to some embodiments said predisposition is due to previous injury or due to family history.

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

The term "brain injury" also refers to subclinical brain injury, spinal cord injury, and anoxic-ischemic brain injury. The term "subclinical brain injury" (SCI) refers to brain injury without overt clinical evidence of brain injury. A lack of clinical evidence of brain injury when brain injury actually exists could result from degree of injury, type of injury, level of consciousness, medications particularly sedation and anesthesia. Many of these origins can lead to Chronic Traumatic Encephalopathy (CTE).

As employed herein, the term "traumatic brain injury" shall mean a brain injury resulting from direct or indirect shock load or loads applied to the brain causing it to move rapidly and unnaturally within a patient's skull and shall expressly include, but not be limited to, brain injuries caused by: (a) objects penetrating the skull, such as, bullets, arrows, and other physical objects which pass through the skull and enter the brain, (b) impact loads applied to the head or other portions of the patient's body, (c) surgically induced trauma, (d) explosions, such as might exist in warfare, through impacting of grenades, bombs, and other explosives, which cause substantial tremors in the earth in relatively-close proximity to where an individual is standing, as well as similar tremors created by nonexplosive means, such as sports injuries, vehicular accidents, collapse of buildings and earthquakes, for example. The results of traumatic brain injury may be of various types, but in each instance, will involve temporary or permanent reduction in the ability of the brain to function normally and may cause death.

One of the consequences of a traumatic brain injury frequently is the generation of inflammation within the brain as the shock to the brain serves to increase the permeability of the endothelial cells, thereby permitting loss of fluids from the vascular structure into the brain. Such a leakage frequently occurs due to the increased porosity of the blood vessels resulting from the trauma, thereby causing blood serum to leak through the vessels into the

brain area. As this builds up, this can generate inflammation and swelling of the brain, which may require surgical intervention.

Clinically, traumatic brain injury can be rated as mild, moderate or severe based on TBI variables that include duration of loss of consciousness (LOC), Glasgow Coma Score (GCS) and post-traumatic stress amnesia.

As used herein, "secondary brain trauma" refers to damage to the brain of a patient post-acute brain injury, i.e., during the secondary injury phase of a TBI.

"Chronic traumatic encephalopathy (CTE)" is a neurodegenerative disease that is most often identified in postmortem autopsies of individuals exposed to repetitive head impacts, such as boxers and football players. The neuropathology of CTE is characterized by the accumulation of hyperphosphorylated tau protein in a pattern that is unique from that of other neurodegenerative diseases, including Alzheimer's disease. The clinical features of CTE are often progressive, leading to dramatic changes in mood, behavior, and cognition, frequently resulting in debilitating dementia. In some cases, motor features, including Parkinsonism, can also be present.

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

"Stroke" refers to the destruction of brain tissue as a result of intracerebral hemorrhage or infarction. Stroke is a leading cause of death in the developed world. It may be caused by reduced blood flow and death of tissues in one area of the brain (infarction). Causes of strokes include blood clots that form in the blood vessels in the brain (thrombus) and blood clots or pieces of atherosclerotic plaque or other material that travel to the brain from another location (emboli). Bleeding (hemorrhage) within the brain may also cause symptoms that mimic stroke.

"Alzheimer's disease (AD)" is a very common yet irreversible, progressive brain disease that slowly destroys memory and thinking skills, and eventually the ability to carry out the simplest tasks. AD is the most common cause of dementia among older people

causing the loss of cognitive functioning thinking, remembering, and reasoning to such an extent that it interferes with a person's daily life and activities. Estimates vary, but experts suggest that as many as 5.1 million Americans may have AD. Currently brain imaging of people with, and those with a family history, of AD or its earlier stage, amnesic mild cognitive impairment (MCI), are beginning to detect changes in the brain. The clinical dementia of AD is coupled with a distinct pathology of senile plaques. AD is characterized by abnormal amyloid beta accumulation and deposition in brain parenchyma and cerebral capillaries, which leads to blood-brain barrier (BBB) disruption.

As used herein, "chronic brain injury" refers to a subject who has suffered a brain injury from three days post injury until at least 12 months previously yet continues to present symptoms of brain injury.

As used herein, "sub-acute brain injury" refers to a subject who has suffered a brain injury from about 2-5 days post injury.

"Conscious", as used herein, has the conventional meaning, as set forth in Plum, et al., The Diagnosis of Stupor and Coma, CNS Series, Philadelphia:Davis (1982), which is hereby incorporated by reference. Conscious patients include those who have a capacity for reliable, reproducible, interactive behavior evidencing awareness of self or the environment. Conscious patients include patients who recover consciousness with less severe brain injury but who, because of their impaired cognitive function, do not reach independent living. Conscious patients do not include those who exhibit wakefulness but lack interaction (e.g., those deemed to be in a persistent vegetative state).

The subject who is conscious after exposure to a head trauma may be asymptomatic of any visible symptoms of traumatic brain injury. Conversely, the subject may exhibit various symptoms of brain injury and cognitive dysfunction.

This is in contrast to a subject who is unconscious at the time of the obtaining, as indicated by conditions such as a concussion or intracranial hemorrhage (e.g. intra-axial hematoma, epidural hematoma, and subdural hematoma).

The phrase "brain injury status" includes any distinguishable manifestation of the condition, including not having brain injury. For example, brain injury status includes,

without limitation, the presence or absence of brain injury in a patient, the risk of developing brain injury, the stage or severity of brain injury, the progress of brain injury (e.g., progress of brain injury over time) and the effectiveness or response to treatment of brain injury (e.g., clinical follow up and surveillance of brain injury after treatment). Based on this status, further procedures may be indicated, including additional diagnostic tests or therapeutic procedures or regimens.

The "spinal cord injury" refers to a condition in which the spinal cord receives compression/detrition due to a vertebral fracture or dislocation to cause dysfunction. As used herein, the term "anoxic-ischemic brain injury" refers to deprivation of oxygen supply to brain tissue resulting in compromised brain function and includes cerebral hypoxia. For example, anoxic-ischemic brain injury includes focal cerebral ischemia, global cerebral ischemia, hypoxic hypoxia (i.e., limited oxygen in the environment causes reduced brain function, such as with divers, aviators, mountain climbers, and fire fighters, all of whom are at risk for this kind of cerebral hypoxia), obstructions in the lungs (e.g., hypoxia resulting from choking, strangulation, the crushing of the windpipe).

The term "brain injury biomarker" (BIB), "brain injury biomarker protein", "brain injury biomarker peptide", brain injury biomarker polypeptide" and the like refer to a protein, including those described herein, that can be used in a method of the present invention, e.g., to diagnose brain injury in a patient. Brain injury biomarker proteins include, but are not limited to, SNCB, GFAP, S100B, MT3, ICAM5, BDNF, and/or NSE. The term also includes other brain injury biomarker proteins known in the art including neurogranin (NRGN), myelin basic protein (MBP), PAD-2, tubulin beta-4B chain, tubulin alpha- IB chain, CNPase, PPIA, Septin-7, Elongation factor l-alpha2, TPPP, TPPP3, Ermin Isoform 2, NDRG2 Isoform 2, astrotactin 1 (ASTN1), brain angiogenesis inhibitor 3 (BAD); carnosine dipeptidase 1 (CNDP 1); ERMTN; glutamate receptor metabotropic 3 (GRM3); kelch-like protein 32 (KLH32); melanoma antigen family E,2 (MAGE2); neuregulin 3 (NRG3); oligodendrocyte myelin glycoprotein (OMG); solute carrier family 39 (zinc transporter); reticulon 1 (RTN1); and peptidylarginine deiminase (types 1-4 and 6) (PAD).

In addition, the term "brain injury biomarkers" also includes the isoforms and/or post-translationally modified forms of any of the foregoing. The present invention contemplates

the detection, measurement, quantification, determination and the like of both unmodified and modifided (e.g., citrullination or other post-translational modification) proteins/polypeptides/peptides as well as autoantibodies to any of the foregoing. In certain embodiments, it is understood that reference to the detection, measurement, determination, and the like, of a biomarker refers detection of the protein/polypeptide/peptide (modified and/or unmodified). In other embodiments, reference to the detection, measurement, determination, and the like, of a biomarker refers detection of autoantibodies of the protein/polypeptide/peptide.

As used herein, the term "comparing" refers to making an assessment of how the reactivity of antibodies in a sample from a patient relates to the reactivity of the corresponding antibodies in a standard or control sample. For example, "comparing" may refer to assessing whether the reactivity of antibodies from a sample of a patient to one or more antigens is the same as, more or less than, or different from the corresponding reactivity of antibodies from the standard or control sample. More specifically, the term may refer to assessing whether the reactivity of antibodies of a sample from a patient to one or more antigens is the same as, more or less than, different from or otherwise corresponds (or not) to a predefined reactivity of antibodies that correspond to, for example, a patient having subclinical brain injury (SCI), not having SCI, is responding to treatment for SCI, is not responding to treatment for SCI, is/is not likely to respond to a particular SCI treatment, or having/not having another disease or condition.

As used herein, the terms "indicates" or "correlates" (or "indicating" or "correlating," or "indication" or "correlation," depending on the context) in reference to a parameter, e.g., a modulated reactivity of antibodies of a sample from a patient, may mean that the patient has brain injury. In specific embodiments, the parameter may comprise the reactivity of antibodies to one or more antigens of the present invention. A particular reactivity of antibodies to one or more antigens may indicate that a patient has brain injury (i.e., correlates to a patient having brain injury). In other embodiments, a particular reactivity of antibodies to one or more antigens may be correlated to a patient being unaffected (i.e., indicates a patient does not have brain injury. In certain embodiments, "indicating," or "correlating," as used according to the present invention, may be by any linear or non-linear method of quantifying the relationship between levels of reactivity of antibodies to a standard, control or comparative value for the assessment of the diagnosis, prediction of

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

According to some embodiments, monitoring the progression of brain injury is conducted at 7-20 day time point post injury, where the neural circulatory reconnections begin to occur. According to some embodiments, the risk of damage to the neural circulatory system is predicted.

The terms "patient," "individual," or "subject" are used interchangeably herein, and refer to a mammal, particularly, a human. The patient may have mild, intermediate or severe disease. The patient may be treatment naive, responding to any form of treatment, or refractory. The patient may be an individual in need of treatment or in need of diagnosis based on particular symptoms or family history. In some cases, the terms may refer to treatment in experimental animals, in veterinary application, and in the development of animal models for disease, including, but not limited to, rodents including mice, rats, and hamsters; and primates.

The term “healthy control” as used herein refers to a healthy individual; a baseline from the same individual, a plurality of healthy individuals, a data set or value corresponding to or obtained from a healthy individual or a plurality of healthy individuals.

The term "Extended Glasgow Outcome Scale (GOSE)" as used herein categorizes functional disability after TBI on a scale of 1-8, where 1=Dead and 8=Upper Good Recovery. The functional disability is defined as a GOSE score of <8.

The terms "measuring", "detecting" and "determining" are used interchangeably throughout, and refer to methods which include obtaining a patient sample and detecting reactivity of antibodies in a sample. In some embodiments, the terms refer to obtaining a patient sample and detecting the reactivity of antibodies in the sample to one or more antigens. Measuring can be accomplished by methods known in the art and those further described herein.

The terms "sample," "patient sample," "biological sample," and the like, encompass a variety of sample types obtained from a patient, individual, or subject and can be used in a diagnostic or monitoring assay. The patient sample may be obtained from a healthy subject, a diseased patient or a patient having associated symptoms of brain injury. Moreover, a sample obtained from a patient can be divided and only a portion may be used for diagnosis. Further, the sample, or a portion thereof, can be stored under conditions to maintain sample for later analysis. The definition specifically encompasses blood and other liquid samples of biological origin (including, but not limited to, peripheral blood, serum, plasma, cerebrospinal fluid, urine, saliva, stool and synovial fluid). In a specific embodiment, a sample comprises a blood sample. In another embodiment, a serum sample is used. The definition also includes samples that have been manipulated in any way after their procurement, such as by centrifugation, filtration, precipitation, dialysis, chromatography, treatment with reagents, washed, or enriched for certain cell populations. The terms further encompass a clinical sample, and also include cells in culture, cell supernatants, tissue samples, organs, and the like. Samples may also comprise fresh-frozen and/or formalin-fixed, paraffin-embedded tissue blocks, such as blocks prepared from clinical or pathological biopsies, prepared for pathological analysis or study by immunohistochemistry.

The samples may be tested immediately after collection, after storage at 4 degrees, -20 degrees, or -80 degrees Celsius. After storage for 24 hours, 1 week, 1 month, 1 year, 10 years or up to 30 years.

Various methodologies of the instant invention include a step that involves comparing a value, level, feature, characteristic, property, etc. to a "suitable control," referred to interchangeably herein as an "appropriate control" or a "control sample." A "suitable control," "appropriate control" or a "control sample" is any control or standard familiar to one of ordinary skill in the art useful for comparison purposes. In one embodiment, a "suitable control" or "appropriate control" is a value, level, feature, characteristic, property, etc., determined in a cell, organ, or patient, e.g., a control or normal cell, organ, or patient, exhibiting, for example, normal traits. For example, the reactivity of antibodies in a sample from an unaffected individual (UI) or a normal control individual (NC) (both terms are used interchangeably herein). In another embodiment, a "suitable control" or "appropriate control" is a value, level, feature, characteristic, property, etc. determined prior to

performing a therapy (e.g., an brain injury treatment) on a patient. In yet another embodiment, a transcription rate, mRNA level, translation rate, protein level, biological activity, cellular characteristic or property, genotype, phenotype, etc., can be determined prior to, during, or after administering a therapy into a cell, organ, or patient. In a further embodiment, a "suitable control" or "appropriate control" is a predefined value, level, feature, characteristic, property, etc. A "suitable control" can be a profile or pattern of reactivity of antibodies to at least one antigen that correlates to brain injury, to which a patient sample can be compared. The patient sample can also be compared to a negative control, i.e., a profile that correlates to not having brain injury.

Antigen probes to be used in the assays of the invention may be purified or synthesized using methods well known in the art. For example, an antigenic protein or peptide may be produced using known recombinant or synthetic methods, including, but not limited to, solid phase (e.g. Boc or f-Moc chemistry) and solution phase synthesis methods (Stewart and Young, 1963; Meienhofer, 1973; Schroder and Lupke, 1965; Sambrook et al., 2001). One of skill in the art will possess the required expertise to obtain or synthesize the antigen probes of the invention. Some of the antigen probes are also commercially available, e.g. from Sigma (St. Louis, Mo., USA), Prospec (Ness-Ziona, Israel), Abnova (Taipei City, Taiwan), Matreya LLC (Pleasant Gap, Pa., USA), Avanti Polar Lipids (Alabaster, Ala., USA), Calbiochem (San Diego, Calif., USA), Chemicon (Temecula, Calif., USA), GeneTex (San Antonio, Tex., USA), Novus Biologicals (Littleton, Colo., USA) Assay Designs (Ann Arbor, Mich., USA), ProSci Inc. (Poway, Calif., USA), EMD Biosciences (San Diego, Calif., USA), Cayman Chemical (Ann Arbor, Mich., USA), HyTest (Turku, Finland), Meridian Life Science (Memphis, Tenn. USA) and Biodesign International (Saco, Me., USA), as detailed herein below.

It should be noted, that the invention utilizes antigen probes as well as homologs, fragments, partial sequences, mutant forms, modified forms and derivatives thereof, as long as these homologs, fragments, partial sequences, mutant forms, modified forms and derivatives are immunologically cross-reactive with these antigen probes. The term "immunologically cross-reactive" as used herein refers to two or more antigens that are specifically bound by the same antibody. The term "homolog" as used herein refers to a peptide which having at least 70%, at least 75%, at least 80%, at least 85% or at least 90% identity to the antigen's amino acid sequence. Cross-reactivity can be determined by any of

a number of immunoassay techniques, such as a competition assay (measuring the ability of a test antigen to competitively inhibit the binding of an antibody to its known antigen).

The term "peptide" typically refers to a polypeptide of up to about 50 amino acid residues in length. According to particular embodiments, the antigenic peptides of the invention may be 10-50 amino acids in length and are typically about 10-30 or about 15-25 amino acids in length.

The term encompasses native peptides (either degradation products, synthetically synthesized peptides, or recombinant peptides), peptidomimetics (typically, synthetically synthesized peptides), and the peptide analogues peptoids and semipeptoids, and may have, for example, modifications rendering the peptides more stable while in a body or more capable of penetrating into cells. Such modifications include, but are not limited to: N-terminus modifications; C-terminus modifications; peptide bond modifications, backbone modifications; and residue modifications.

The peptide antigens of the invention may be used having a terminal carboxy acid, as a carboxy amide, as a reduced terminal alcohol or as any pharmaceutically acceptable salt, e.g., as metal salt, including sodium, potassium, lithium or calcium salt, or as a salt with an organic base, or as a salt with a mineral acid, including sulfuric acid, hydrochloric acid or phosphoric acid, or with an organic acid e.g., acetic acid or maleic acid. According to some embodiments, the peptide antigens of the invention are BSA-conjugated peptides.

Functional derivatives consist of chemical modifications to amino acid side chains and/or the carboxyl and/or amino moieties of said peptides. Such derivatized molecules include, for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as chemical derivatives are those polypeptides, which contain one or more naturally occurring or modified amino acid derivatives of the twenty standard amino acid residues. For example: 4-hydroxyproline may be substituted for proline; 5-hydroxylysine

may be substituted for lysine; 3-methylhistidine may be substituted for histidine; homoserine may be substituted or serine; and ornithine may be substituted for lysine.

The amino acid residues described herein are in the "L" isomeric form, unless otherwise indicated. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the peptide substantially retains the desired antibody specificity.

Suitable analogs may be readily synthesized by now-standard peptide synthesis methods and apparatus or recombinant methods. All such analogs will essentially be based on the antigens of the invention as regards their amino acid sequence but will have one or more amino acid residues deleted, substituted or added. When amino acid residues are substituted, such conservative replacements which are envisaged are those which do not significantly alter the structure or antigenicity of the polypeptide. For example basic amino acids will be replaced with other basic amino acids, acidic ones with acidic ones and neutral ones with neutral ones. In addition to analogs comprising conservative substitutions as detailed above, analogs comprising non-conservative amino acid substitutions are further contemplated, as long as these analogs are immunologically cross reactive with a peptide antigen of the invention.

In other aspects, there are provided nucleic acids encoding these peptides, vectors comprising these nucleic acids and host cells containing them. These nucleic acids, vectors and host cells are readily produced by recombinant methods known in the art (see, e.g., Sambrook et al., 2001). For example, an isolated nucleic acid sequence encoding an antigen of the invention can be obtained from its natural source, either as an entire (i.e., complete) gene or a portion thereof. A nucleic acid molecule can also be produced using recombinant DNA technology (e.g., polymerase chain reaction (PCR) amplification, cloning) or chemical synthesis. Nucleic acid sequences include natural nucleic acid sequences and homologs thereof, including, but not limited to, natural allelic variants and modified nucleic acid sequences in which nucleotides have been inserted, deleted, substituted, and/or inverted in such a manner that such modifications do not substantially interfere with the nucleic acid molecule's ability to encode a functional peptide of the present invention.

"Functionally equivalent variants" of the polypeptide or peptide antigens of the invention as used herein are polypeptides or peptides with partial sequence homology, polypeptides or peptides having one or more specific conservative and/or non-conservative amino acid changes and polypeptide or peptide conjugates which do not alter the biological or structural properties of the polypeptide or peptide.

In terms of "functional analogues", it is well understood by those skilled in the art, that inherent in the definition of a biologically functional polypeptide or peptide analogue is the concept that there is a limit to the number of changes that may be made within a defined portion of the molecule and still result in a molecule with an acceptable level of equivalent biological activity. A plurality of distinct polypeptides or peptides with different substitutions may easily be made and used in accordance with the invention. It is also understood that certain residues are particularly important to the biological or structural properties of a polypeptide, and such residues may not generally be exchanged.

Functional analogues can be generated by conservative or non-conservative amino acid substitutions. Amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size and the like. Thus, within the scope of the invention, conservative amino acid changes means, an amino acid change at a particular position which is of the same type as originally present; i.e. a hydrophobic amino acid exchanged for a hydrophobic amino acid, a basic amino acid for a basic amino acid, etc. Examples of conservative substitutions include the substitution of non-polar (hydrophobic) residues such as isoleucine, valine, leucine or methionine for another, the substitution of one polar (hydrophilic) residue for another such as between arginine and lysine, between glutamine and asparagine, between glycine and serine, the substitution of one basic residue such as lysine, arginine or histidine for another, or the substitution of one acidic residue, such as aspartic acid or glutamic acid for another, the substitution of a branched chain amino acid, such as isoleucine, leucine, or valine for another, the substitution of one aromatic amino acid, such as phenylalanine, tyrosine or tryptophan for another. Such amino acid changes result in functional analogues in that they do not significantly alter the overall charge and/or configuration of the polypeptide. Examples of such conservative changes are well-known to the skilled artisan and are within the scope of the present invention. Conservative substitution also includes the use of a chemically derivatized residue in place of a non-derivatized residue provided

that the resulting polypeptide is a biologically functional equivalent to the polypeptide antigens.

Therefore, the "citrullinated polypeptides" encompass a polypeptide having an amino acid sequence that differs from the sequences provided herein by one or more conservative amino acid substitutions. The citrullinated polypeptides also encompass a polypeptide having an amino acid sequence that differs from the sequences provided herein by a single mutation, where the single mutation represents a single amino acid deletion, insertion or substitution.

The citrullinated peptides may be made by methods known to those of skill in the art most notably and preferably by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield et al, 65 J. AM. CHEM. ASSOC. 2149 (1964); Merrifield et al, 85 J. AMER. CHEM. SOC. 2149 (1963); and Merrifield et al, 35 INT. J. PEPTIDE PROTEIN RES. 161 -214 (1990)) or synthesis in homogenous solution (METHODS OF ORGANIC CHEMISTRY, E. Wansch (Ed.) Vol. 15, pts. I and II, Thieme, Stuttgart (1987)) to generate synthetic peptides. Citrulline is a post-translationally modified arginine that is created through the process of deimination which is catalyzed by the enzyme peptidylarginine deiminase 4 (PAD-4) that removes a positive charge from arginine and makes the resulting citrulline polar in nature.

In one embodiment, citrullinated peptides can be made from known commercially available sources. In this aspect, the lyophilized protein is reconstituted in an appropriate buffer to which the enzyme peptidylarginine deiminase 4 is added. Alternatively, Ca^{2+} is added to PAD-4 in solution. The solution is allowed to stand at an appropriate temperature for a time sufficient to cause modification of arginine residues to citrulline and thus create a citrullinated protein. The citrullinated protein is then isolated by the removal of the enzyme using a high molecular weight membrane to separate the enzyme or other methods of chromatography. One of skill in the art will understand that the temperature of incubation, buffer condition and time of incubation may vary depending on the protein that is being deiminated (Masson-Bessiere et al, 166 J. IMMUNOL. 4177-4184 (2001)).

The citrullinated proteins may be further isolated and purified by methods selected on the basis of properties revealed by its sequence. Purification can be achieved by protein

purification procedures such as chromatography methods (gel-filtration, ion-exchange and immunoaffinity), by high-performance liquid chromatography (HPLC, RP-HPLC, ion-exchange HPLC, size-exclusion HPLC, high-performance chromatofocusing and hydrophobic interaction chromatography) or by precipitation (immunoprecipitation).

Polyacrylamide gel electrophoresis can also be used to isolate the citrullinated proteins based on the molecular weight of the protein, charge properties and hydrophobicity. The purified citrullinated proteins can be used in further biochemical analyses to establish secondary and tertiary structure which may aid in the design of pharmaceuticals to interact with the protein, alter the protein charge configuration or charge interaction with other proteins or alter its function.

The term “oligonucleotide antigen” as used herein refer to a stretch of contiguous nucleotides of a certain length. Unless otherwise indicated, the term "oligonucleotide antigen" as used herein relates to a nucleotide sequence of between 15 and 40 nucleotides in length, alternatively between 17 and 28 nucleotides in length, or between 18-25 nucleotides in length. In certain embodiments, an oligonucleotide antigen consists of at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 16, or more contiguous nucleotides. Each possibility represents a separate embodiment of the invention. In certain embodiments, an antigen consists of not more than 50, not more than 45, not more than 40, not more than 35, not more than 30, not more than 25, not more than 20, not more than 16, or less contiguous nucleotides. Each possibility represents a separate embodiment of the invention. In certain embodiments, an antigen consists of 10-30, 15-25 or 17-20 contiguous nucleotides. In certain embodiments, an antigen consists of 17, 18, 19 or 20 contiguous nucleotides.

As used herein, the “reactivity of antibodies in a sample” or “reactivity of an antibody in a sample” to “an antigen” or to “a plurality of antigens” refers to the immune reactivity of at least one antibody in the sample to at least one specific antigen selected from the plurality of antigens. The immune reactivity of the antibody to the antigen, i.e. its ability to specifically bind the antigen, may be used to determine the amount of the antibody in the sample. The calculated levels of each one of the tested antibodies in the sample are collectively referred to as the reactivity pattern of the sample to these antigens. The reactivity pattern of the sample reflects the levels of each one of the tested antibodies in the

sample, thereby providing a quantitative assay. In a preferred embodiment, the antibodies are quantitatively determined.

A "significant difference" between reactivity patterns refers, in different embodiments, to a statistically significant difference, or in other embodiments to a significant difference as recognized by a skilled artisan. In another embodiment, a significant difference between the reactivity pattern of the sample obtained from the subject compared to the control reactivity pattern is an indication that the subject is afflicted with brain injury. In specific embodiments, up-regulation or higher reactivity of the reactivity of an antibody in a sample to an antigen refers to an increase (i.e., elevation) of about at least two, about at least three, about at least four, or about at least five times higher (i.e., greater) than the reactivity levels of the antibody to the antigen in the control. In another embodiment, down-regulation or lower reactivity of the reactivity of an antibody in a sample to an antigen refers to a decrease (i.e., reduction) of about at least two, about at least three, about at least four, or about at least five times lower than the reactivity levels of the antibody to the antigen in the control.

According to some embodiments, the at least one oligonucleotide antigen is an oligonucleotide sequence comprising at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 contiguous adenine nucleotides. According to another embodiment, the oligonucleotide sequence comprises at most 20 contiguous adenine nucleotides. According to additional embodiments, the at least one oligonucleotide antigen is an oligonucleotide sequence comprising at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 contiguous thymine nucleotides. According to another embodiment, the oligonucleotide sequence comprises at most 20 contiguous thymine nucleotides.

According to additional embodiments, the at least one oligonucleotide antigen is an oligonucleotide sequence comprising at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 contiguous cytosine nucleotides. According to another embodiment, the oligonucleotide sequence comprises at most 20 contiguous cytosine nucleotides. According to additional embodiments, the at least one oligonucleotide antigen is an oligonucleotide sequence comprising 5-17, 6-17, 7-17, 8-17, 9-17, 10-17, 11-17, 12-17, 13-17, 14-17, 15-17, 16-17, or at most 17 contiguous guanine nucleotides.

According to some embodiments, the at least one antigen is selected from the group consisting of SEQ ID NOs: 1-115, isoforms thereof, post-translationally modified forms thereof, fragments thereof; or combinations of any of the foregoing.

According to some embodiments, the antigens are selected from proteins, peptides, oligonucleotide antigens, or any combinations thereof.

It should be understood each antigen according to the present invention may be bound by IgM antibodies and/or IgG antibodies found or isolated from a sample obtained or derived from the tested subject. Since the relative amounts of IgM antibodies and IgG antibodies against a certain epitope or antigen naturally change over the course of time, each antigen according to the present invention may be bound by IgM antibodies, IgG antibodies or both. In certain embodiments, the reactivity of antibodies means the reactivity of IgG antibodies. In certain embodiments, the reactivity of antibodies means the reactivity of IgM antibodies. According to another embodiment, the significantly higher reactivity of the antibodies in the sample means increased IgG reactivity. According to another embodiment, the significantly higher reactivity of the antibodies in the sample comprises increased IgM reactivity.

According to another embodiment, the increased IgM reactivity is of at least one antigen selected from the group consisting of SEQ ID NOs: 1-115, isoforms thereof, post-translationally modified forms thereof, or combinations of any of the foregoing.

According to another embodiment, the increased IgG reactivity is of at least one antigen selected from the group consisting of SEQ ID NOs: 1-115, isoforms thereof, post-translationally modified forms thereof, or combinations of any of the foregoing.

In certain embodiments, the increased IgM and IgG reactivity is of at least one antigen selected from the group consisting of SEQ ID NOs: 1-115, isoforms thereof, post-translationally modified forms thereof, or combinations of any of the foregoing. In certain embodiments, the increased IgM reactivity is of at least one antigen selected from the group consisting of SEQ ID NOs: 1-115, isoforms thereof, post-translationally modified forms thereof, fragments thereof, or combinations of any of the foregoing. Each possibility represents a separate embodiment of the invention.

It should be understood that in order to perform the methods of the present invention, samples obtained or derived from subjects must comprise antibodies produced by the subject himself. Therefore, samples may be obtained or derived from any tissue, organ or liquid naturally comprising at least a subset of the subject's antibodies. In certain embodiments, the sample obtained from the subject is a biological fluid. According to some embodiments, the sample is selected from the group consisting of plasma, serum, blood, cerebrospinal fluid, synovial fluid, sputum, urine, saliva, tears, lymph specimen, or any other biological fluid known in the art. Each possibility represents a separate embodiment of the invention. According to certain embodiments, the sample obtained from the subject is selected from the group consisting of serum, plasma and blood. According to one embodiment, the sample is a serum sample. Methods for obtaining and isolating appropriate samples are well within the purview of the skilled artisan.

According to certain embodiments of the methods of the present invention, the control is selected from the group consisting of a sample from at least one healthy individual, base line of the same subject, a panel of control samples from a set of healthy individuals, and a stored set of data from healthy individuals. Each possibility represents a separate embodiment of the invention. Typically, a healthy individual is a subject not afflicted with brain injury.

In particular embodiments, the significant difference is determined using a cutoff of a positive predictive value (PPV) of at least 85%, preferably at least 90%. Determining a PPV for a selected marker (e.g., an antigen) is well known to the ordinarily skilled artisan and is exemplified in the methods described below. Typically, positivity for an antigen is determined if it detected above 10% of the subjects in a specific study subgroup using a selected cutoff value, such as PPV $\geq$90%. For example, antigen *i* is determined to specifically characterize group A if it detected at least 10% of the subjects in group A with a PPV $\geq$90% when compared to a different test group B. Subjects in group A that are above the cutoff of PPV $\geq$90% for antigen *i* are considered to be positive for antigen *i*.

An antibody "directed to" an antigen, as used herein is an antibody which is capable of specific binding to the antigen. Determining the levels of antibodies directed to a plurality of antigens includes measuring the level of each antibody in the sample, wherein

each antibody is directed to a specific antigen of the invention. This step is typically performed using an immunoassay, as detailed herein.

In other embodiments, determining the reactivity of antibodies in the sample to the at least one antigen (and the levels of each one of the tested antibodies in the sample) is performed by a process comprising contacting the sample, under conditions such that a specific antigen-antibody complex may be formed, with at least one antigen (or when a plurality of antigens is used, to an antigen probe set comprising the plurality of antigens), and quantifying the amount of antigen-antibody complex formed for each antigen probe. The amount of antigen-antibody complex is indicative of the level of the tested antibody in the sample (or the reactivity of the sample with the antigen).

In another embodiment the method comprises determining the reactivity of at least one IgG antibody and at least one IgM antibody in the sample to the plurality of antigens. In another embodiment, the method comprises determining the reactivity of a plurality of IgG antibodies and at least one IgM antibody in the sample to the plurality of antigens. In another embodiment, the method comprises determining the reactivity of at least one IgG antibody and a plurality of IgM antibodies in the sample to the plurality of antigens. According to another embodiment, the method comprises determining the reactivity of antibodies in the sample to a plurality of antigens.

Typically, determining the reactivity of antibodies in the sample to at least one antigen is performed using an immunoassay. Advantageously, when a plurality of antigens is used, the plurality of antigens may be used in the form of an antigen array.

Antigen probes and antigen probe sets

According to further embodiments, the invention provides antigen probes and antigen probe sets useful for diagnosing brain injury, as detailed herein.

The invention further provides a plurality of antigens also referred to herein as antigen probe sets. These antigen probe sets comprise a plurality of antigens which are reactive specifically with the sera of subjects having brain injury. According to the principles of the invention, the plurality of antigens may advantageously be used in the form of an antigen

array. According to some embodiments the antigen array is conveniently arranged in the form of an antigen chip.

A "probe" as used herein means any compound capable of specific binding to a component. According to one aspect, the present invention provides an antigen probe set comprising a plurality of antigens selected from the group consisting of: SEQ ID NOs: 1-115, isoforms thereof, post-translationally modified forms thereof, fragments thereof, or combinations of any of the foregoing. According to certain embodiments, the antigen probe set comprises a subset of the antigens of the present invention. In a particular embodiment, the subset of antigens consists of: SEQ ID NOs: 1-24, 27-30, 42, 75, 76, isoforms thereof, post-translationally modified forms thereof, fragments thereof, or combinations of any of the foregoing.

In some embodiments, antigen probe set consists of up to 300 antigens. In some embodiments, the antigen probe set consists of 2-5 antigens.

According to another embodiment, the methods of the present invention comprise determining the reactivity of antibodies in the sample to at least one antigen selected from the group consisting of SEQ ID NO: 1-115, isoforms thereof, post-translationally modified forms thereof, fragments thereof, or combinations of any of the foregoing.

The reactivity of antibodies to the plurality of antigens of the invention may be determined according to techniques known in the art.

Preferably, the plurality of antigens of the methods and kits of the invention comprises a set of the antigens as disclosed herein. Yet in other embodiments, the plurality of antigens (or the antigen probe set) comprises or consists of a subset thereof, e.g. 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 ,26, 27, 28, 29, 30, 31, 32, 33,34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66 or 115 different antigens, each selected from the antigens of the present invention, wherein each possibility represents a separate embodiment of the invention. Such subsets may be selected so as to result in optimal sensitivity and/or specificity of the diagnostic assay.

Antigen probes to be used in the assays of the invention may be synthesized or purified using methods well known in the art.

It should be noted, that the invention utilizes antigen probes as well as homologs, fragments and derivatives thereof, as long as these homologs, fragments and derivatives are immunologically cross-reactive with these antigen probes. The term "f" as used herein refers to two or more antigens that are specifically bound by the same antibody. The term "homolog" as used herein refers to an antigen probes having at least 80%, at least 85% or at least 90% identity to the antigen's sequence or structure. Cross-reactivity can be determined by any of a number of immunoassay techniques, such as a competition assay (measuring the ability of a test antigen to competitively inhibit the binding of an antibody to its known antigen).

The term "fragment" as used herein refers to a portion of an antigen, or antigen analog which remains immunologically cross-reactive with the antigen probes, e.g., to immunospecifically recognize the target antigen. The fragment may have the length of about 80%, about 85%, about 90% or about 95% of the respective antigen.

According to another aspect, the present invention provides an antigen probe set comprising a plurality of antigen probes selected from the group consisting of SEQ ID NOs: 1-115, isoforms thereof, post-translationally modified forms thereof, fragments thereof, or combinations of any of the foregoing.

According to another related aspect, the present invention provides an antigen probe set comprising at least one antigen probe selected from the group consisting of SEQ ID NOs: 1-115, isoforms thereof, post-translationally modified forms thereof, fragments thereof, or combinations of any of the foregoing.

According to another aspect, the present invention provides an article of manufacture comprising the at least one of the antigen probe sets described above.

In certain embodiments, the article of manufacture is in the form of an antigen probe array or in the form of an antigen chip or in the form of a dipstick or in the form of a lateral flow test or any other platform known to those skilled in the art. An "antigen probe array" generally refers to a plurality of antigen probes, either mixed in a single container or

arranges in to or more containers. An "antigen chip" generally refers to a substantially two dimensional surface, onto which a plurality of antigens are attached or adhered. A "dipstick" generally refers to an object, onto which one or a plurality of antigens are attached or adhered, which is dipped into a liquid to perform a chemical test or to provide a measure of quantity found in the liquid. A "lateral flow test" generally refers to devices intended to detect the presence (or absence) of a target analyte in sample (matrix) without the need for specialized and costly equipment. In certain embodiments, the article of manufacture is in the form of a kit.

According to certain embodiments, the kit further comprises means for determining the reactivity of antibodies in a sample to at least one antigen of the plurality of antigens. According to another embodiment, the kit further comprises means for comparing reactivity of antibody in different samples to at least one antigen of the plurality of antigens. According to another embodiment, the kit further comprises instructions for use. For example, the aforementioned means may include reagents, detectable labels and/or containers which may be used for measuring specific binding of antibodies to the antigen probes of the invention. "Means" as used herein may also refer to devices, reagents and chemicals, such as vials, buffers and written protocols or instructions, used to perform biological or chemical assays.

According to another aspect, there is provided use of the at least one antigen selected from the group consisting of: SEQ ID NOs: 1-115, isoforms thereof, post-translationally modified forms thereof, fragments thereof, or combinations of any of the foregoing; for the preparation of a diagnostic kit for diagnosing brain injury in a subject. The diagnostic kit is, in some embodiments, useful for determining the reactivity of antibodies in a sample, thereby determining the reactivity pattern of the sample to the at least one antigen. In some embodiments, a significant difference (e.g., increase) between the reactivity pattern of the sample compared to a reactivity pattern of a control sample is an indication for brain injury.

In other embodiments, the plurality of antigens comprised in the antigen probe set comprises or consists up to 50, 55, 60, 70, 80, 90 or 100 different antigens. In other embodiments, the plurality of antigens comprised in the antigen probe set comprises or consists at least 50, 100, 150, 200 or 500 different antigens.

In other aspects, there are provided nucleic-acid vectors comprising the oligonucleotides of the invention and host cells containing them. These nucleic acids, vectors and host cells are readily produced by recombinant methods known in the art. A poly-nucleic acid molecule can also be produced using recombinant DNA technology (e.g., polymerase chain reaction (PCR) amplification, cloning) or chemical synthesis. Nucleic acid sequences include natural nucleic acid sequences and homologs thereof, including, but not limited to, natural allelic variants and modified nucleic acid sequences in which nucleotides have been inserted, deleted, substituted, and/or inverted in such a manner that such modifications do not substantially interfere with the nucleic acid molecule's ability to perform the methods of the present invention.

According to the invention, the kits comprise a plurality of antigens also referred to herein as antigen probe sets. These antigen probe sets comprising a plurality of antigens are reactive specifically with the sera of subjects having brain injury. In some embodiments, the antigen probe sets can differentiate between sera of subjects having brain injury and normal subject. According to the principles of the invention, the plurality of antigens may advantageously be used in the form of an antigen array. According to some embodiments the antigen array is conveniently arranged in the form of an antigen chip.

In other embodiments, the kit may further comprise means for determining the reactivity of antibodies in a sample to the plurality of antigens. For example, the kit may contain reagents, detectable labels and/or containers which may be used for measuring specific binding of antibodies to the antigen probes of the invention. In a particular embodiment, the kit is in the form of an antigen array.

In some embodiments, the kit comprises means for comparing reactivity patterns of antibodies in different samples to the plurality of antigens. In other embodiments, the kit may further comprise negative and/or positive control samples. For example, a negative control sample may contain a sample from at least one healthy individual (e.g., an individual not-afflicted with brain injury). A positive control may contain a sample from at least one individual afflicted with brain injury, or a subtype of brain injury which is being diagnosed. Other non-limiting examples are a panel of control samples from a set of healthy individuals or diseased individuals, or a stored set of data from control individuals.

Antibodies, samples and immunoassays

Antibodies, or immunoglobulins, comprise two heavy chains linked together by disulfide bonds and two light chains, each light chain being linked to a respective heavy chain by disulfide bonds in a "Y" shaped configuration. Each heavy chain has at one end a variable domain (VH) followed by a number of constant domains (CH). Each light chain has a variable domain (VL) at one end and a constant domain (CL) at its other end, the light chain variable domain being aligned with the variable domain of the heavy chain and the light chain constant domain being aligned with the first constant domain of the heavy chain (CH1). The variable domains of each pair of light and heavy chains form the antigen binding site.

The isotype of the heavy chain (gamma, alpha, delta, epsilon or mu) determines immunoglobulin class (IgG, IgA, IgD, IgE or IgM, respectively). The light chain is either of two isotypes (kappa, κ or lambda, λ) found in all antibody classes.

It should be understood that when the terms "antibody" or "antibodies" are used, this is intended to include intact antibodies, such as polyclonal antibodies or monoclonal antibodies (mAbs), as well as proteolytic fragments thereof such as the Fab or $F(ab')_2$ fragments. Further included within the scope of the invention (for example as immunoassay reagents, as detailed herein) are chimeric antibodies; recombinant and engineered antibodies, and fragments thereof.

Exemplary functional antibody fragments comprising whole or essentially whole variable regions of both light and heavy chains are defined as follows: (i) Fv, defined as a genetically engineered fragment consisting of the variable region of the light chain and the variable region of the heavy chain expressed as two chains; (ii) single-chain Fv ("scFv"), a genetically engineered single-chain molecule including the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker; (iii) Fab, a fragment of an antibody molecule containing a monovalent antigen-binding portion of an antibody molecule, obtained by treating whole antibody with the enzyme papain to yield the intact light chain and the Fd fragment of the heavy chain, which consists of the variable and CH1 domains thereof: (iv) Fab', a fragment of an antibody molecule containing a monovalent antigen-binding portion of an antibody molecule, obtained by treating whole antibody with the enzyme pepsin, followed by reduction (two Fab' fragments are obtained

per antibody molecule); and (v) F(ab')2, a fragment of an antibody molecule containing a monovalent antigen-binding portion of an antibody molecule, obtained by treating whole antibody with the enzyme pepsin (i.e., a dimer of Fab' fragments held together by two disulfide bonds).

The term "antigen" as used herein is a molecule or a portion of a molecule capable of being bound by an antibody. The antigen is typically capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. An antigen may have one or more epitopes. The specific reaction referred to above is meant to indicate that the antigen will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies which may be evoked by other antigens. An "antigenic peptide" is a peptide which is capable of specifically binding an antibody.

In another embodiment, detection of the capacity of an antibody to specifically bind an antigen probe may be performed by quantifying specific antigen-antibody complex formation. The term "specifically bind" as used herein means that the binding of an antibody to a specific antigen probe is not affected by the presence of non-related molecules.

In certain embodiments, the method of the present invention is performed by determining the capacity of an antigen of the invention to specifically bind antibodies of the IgG isotype, or, in other embodiments, antibodies of the IgM, isolated from a subject.

Methods for obtaining suitable antibody-containing biological samples from a subject are well within the ability of those of skill in the art. Typically, suitable samples comprise whole blood and products derived therefrom, such as plasma and serum. In other embodiments, other antibody-containing samples may be used, e.g. CSF, urine and saliva samples.

Numerous well known fluid collection methods can be utilized to collect the biological sample from the subject in order to perform the methods of the invention.

In accordance with the present invention, any suitable immunoassay can be used with the subject antigens. Such techniques are well known to the ordinarily skilled artisan and have been described in many standard immunology manuals and texts. In certain preferable

embodiments, determining the capacity of the antibodies to specifically bind the antigen probes is performed using an antigen probe array-based method. Preferably, the array is incubated with suitably diluted serum of the subject so as to allow specific binding between antibodies contained in the serum and the immobilized antigen probes, washing out unbound serum from the array, incubating the washed array with a detectable label-conjugated ligand of antibodies of the desired isotype, washing out unbound label from the array, and measuring levels of the label bound to each antigen probe.

In various embodiments, the method of the present invention further comprises diluting the sample before performing the determining step. In one embodiment, the sample is diluted 1:2, for instance, using PBS. In another embodiment, the sample is diluted 1:4, 1:6, 1:8, 1:15, 1:20, 1:50, or preferably 1:10. Each possibility represents a separate embodiment of the present invention. In another embodiment, the sample is diluted in the range of times 2 - times 10. In another embodiment, the sample is diluted in the range of times 4 - times 10. In another embodiment, the sample is diluted in the range of times 6 - times 10. In another embodiment, the sample is diluted in the range of times 8 - times 10.

The antigen chip

Antigen microarrays are used for the high-throughput characterization of the immune response (Robinson et al., 2002, *Nat Med* 8, 295-301), and have been used to analyze immune responses in vaccination and in autoimmune disorders (Robinson et al., 2002; Robinson et al., 2003, *Nat Biotechnol.* 21, 1033-9; Quintana et al., 2004; Kanter et al., 2006, *Nat Med* 12, 138-43). It has been hypothesized, that patterns of multiple reactivities may be more revealing than single antigen-antibody relationships (Quintana et al., 2006, *Lupus* 15, 428-30) as shown in previous analyses of autoimmune repertoires of mice (Quintana et al., 2004; Quintana et al., 2001, *J Autoimmun* 17, 191-7) and humans (Merbl et al., 2007, *J Clin Invest* 117, 712-8; Quintana et al., 2003, *J Autoimmun* 21, 65-75) in health and disease. Thus, autoantibody repertoires have the potential to provide both new insights into the pathogenesis of the disease and to serve as immune biomarkers (Cohen, 2007, *Nat Rev Immunol.* 7, 569-74) of the disease process.

According to some aspects the methods of the present invention may be practiced using antigen arrays as disclosed in WO 02/08755 and U.S. 2005/0260770, the contents of

which are incorporated herein by reference. WO 02/08755 is directed to a system and an article of manufacture for clustering and thereby identifying predefined antigens reactive with undetermined immunoglobulins of sera derived from patient subjects in need of diagnosis of disease or monitoring of treatment. Further disclosed are diagnostic methods, and systems useful in these methods, employing the step of clustering a subset of antigens of a plurality of antigens, the subset of antigens being reactive with a plurality of antibodies being derived from a plurality of patients, and associating or disassociating the antibodies of a subject with the resulting cluster.

U.S. Pat. App. Pub. No. 2005/0260770 discloses an antigen array system and diagnostic uses thereof. The application provides a method of diagnosing an immune disease, particularly diabetes type 1, or a predisposition thereto in a subject, comprising determining a capacity of immunoglobulins of the subject to specifically bind each antigen probe of an antigen probe set. The teachings of the disclosures are incorporated in their entirety as if fully set forth herein.

In other embodiments, various other immunoassays may be used, including, without limitation, enzyme-linked immunosorbent assay (ELISA), flow cytometry with multiplex beads (such as the system made by Luminex), surface plasmon resonance (SPR), elipsometry, and various other immunoassays which employ, for example, laser scanning, light detecting, photon detecting via a photo-multiplier, photographing with a digital camera based system or video system, radiation counting, fluorescence detecting, electronic, magnetic detecting and any other system that allows quantitative measurement of antigen-antibody binding.

Various methods have been developed for preparing arrays suitable for the methods of the present invention. State-of-the-art methods involves using a robotic apparatus to apply or "spot" distinct solutions containing antigen probes to closely spaced specific addressable locations on the surface of a planar support, typically a glass support, such as a microscope slide, which is subsequently processed by suitable thermal and/or chemical treatment to attach antigen probes to the surface of the support. First, the glass surface is activated by a chemical treatment that leaves a layer of reactive groups such as epoxy groups on the surface, which bind covalently any molecule containing free amine or thiol groups. Suitable supports may also include silicon, nitrocellulose, paper, cellulosic supports and the like.

Preferably, each antigen probe, or distinct subset of antigen probes of the present invention, which is attached to a specific addressable location of the array is attached independently to at least two, more preferably to at least three separate specific addressable locations of the array in order to enable generation of statistically robust data.

According to additional embodiments, the antigen probe set comprises at least 5, at least 25, at least 100, at least 150, at least 200, at least 250, at least 300 or more antigens, including one or a plurality of the antigens provided by the present invention.

In addition to antigen probes of the invention, the array may advantageously include control antigen probes or other standard chemicals. Such control antigen probes may include normalization control probes. The signals obtained from the normalization control probes provide a control for variations in binding conditions, label intensity, "reading" efficiency and other factors that may cause the signal of a given binding antibody-probe ligand interaction to vary. For example, signals, such as fluorescence intensity, read from all other antigen probes of the antigen probe array are divided by the signal (e.g., fluorescence intensity) from the normalization control probes thereby normalizing the measurements. Normalization control probes can be bound to various addressable locations on the antigen probe array to control for spatial variation in antibody-ligand probe efficiency. Preferably, normalization control probes are located at the corners or edges of the array to control for edge effects, as well as in the middle of the array.

The labeled antibody ligands may be of any of various suitable types of antibody ligand. Preferably, the antibody ligand is an antibody which is capable of specifically binding the Fc portion of the antibodies of the subject used. For example, where the antibodies of the subject are of the IgM isotype, the antibody ligand is preferably an antibody capable of specifically binding to the Fc region of IgM antibodies of the subject.

The ligand of the antibodies of the subject may be conjugated to any of various types of detectable labels. Preferably the label is a fluorophore, most preferably Cy3. Alternately, the fluorophore may be any of various fluorophores, including Cy5, Dy5, fluorescein isothiocyanate (FITC), phycoerythrin (PE), rhodamine, Texas red, and the like. Suitable fluorophore-conjugated antibodies specific for antibodies of a specific isotype are widely available from commercial suppliers and methods of their production are well established.

Antibodies of the subject may be isolated for analysis of their antigen probe binding capacity in any of various ways, depending on the application and purpose. While the subject's antibodies may be suitably and conveniently in the form of blood serum or plasma or a dilution thereof (e.g. 1:10 dilution), the antibodies may be subjected to any desired degree of purification prior to being tested for their capacity to specifically bind antigen probes. The method of the present invention may be practiced using whole antibodies of the subject, or antibody fragments of the subject which comprises an antibody variable region.

Combination measurement of the levels of one or more antibodies and one or more biomarkers in the sample obtained from the subject

The present invention is based, at least in part, on the discovery that a combination measurement of the levels of one or more antibodies and one or more biomarkers in the sample obtained from the subject can measure both the real-time background physiology of the subject and the status of the acute event.

In a patient with Brain Injury, the response to injury and the recovery process from the injury is dependent upon a combination of the nature of the injury and the state of the individual prior to injury. Patients that are injured on top of a 'healthy' background will likely have a better (faster, more complete) recovery profile than patients that are injured on a 'sick' or 'previously injured' background.

Determination of the autoantibody profile of a patient can be used as a surrogate measurement of the state of the patient prior to brain injury and determination of the levels of circulating antigen shortly after injury can be used as a surrogate measurement of the nature/degree of injury. Algorithms that combine the information about the state of the patient prior to injury and the nature/degree of the injury can be used in order to predict outcomes.

Determination of the autoantibody profile can be performed using any platform where antigens are bound to a surface, circulating antibodies bind to the antigen and are detected with a tagged secondary antibody. Determination of the circulating antigen profile can be done in any ELISA type sandwich assay format which includes a capture antibody and a detection antibody.

The platforms used for antibody and antigen detection may be independent (eg. iCHIP for autoantibody, MSD ELISA for antigens or any relevant ELISA based platform) or may be combined into a single platform to simultaneously measure both circulating autoantibody and antigen. This can be done by printing an iCHIP with both relevant antigens and capture antibodies, contacting serum with the printed surface such that circulating antibodies will bind to the surface bound antigen, and circulating antigens will bind to the surface bound capture antibodies. Detection can be with a cocktail of secondary and detection antibodies.

In the case where there is a need to measure autoantibodies to the same antigen that is informative about the disease state, these measurements can we done in two separate chambers. The data from multiple tests can be combined for the purpose of an algorithmic analysis to finally predict the status of the patient.

Kits for the Detection of Biomarkers

In another aspect, the present invention provides kits for qualifying brain injury status, which kits are used to detect the biomarkers described herein. In a specific embodiment, the kit is provided as an ELISA kit comprising antibodies to the biomarkers of the present invention including, but not limited to, glial fibrillary acidic protein (GFAP) and Synuclein beta (Sncb).

In an alternative embodiment, the panel of biomarkers comprises BDNF, GFAP, MT3 and SNCB. In another embodiment, the panel of biomarkers comprises BDNF, GFAP, NRGN and SNCB. In a further embodiment, the panel of biomarkers comprises BDNF, ICAM5, MT3 and SNCB.

The ELISA kit may comprise a solid support, such as a chip, microtiter plate (e.g., a 96-well plate), bead, or resin having biomarker capture reagents attached thereon.

The kit may further comprise a means for detecting the biomarkers, such as antibodies, and a secondary antibody-signal complex such as horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody and tetramethyl benzidine (TMB) as a substrate for HRP.

The kit may be provided as an immuno-chromatography strip comprising a membrane on which the antibodies are immobilized, and a means for detecting, e.g., gold particle

bound antibodies, where the membrane, includes NC membrane and PVDF membrane. The kit may comprise a plastic plate on which a sample application pad, gold particle bound antibodies temporally immobilized on a glass fiber filter, a nitrocellulose membrane on which antibody bands and a secondary antibody band are immobilized and an absorbent pad are positioned in a serial manner, so as to keep continuous capillary flow of blood serum.

Data analysis

Advantageously, the methods of the invention may employ the use of learning and pattern recognition analyzers, clustering algorithms and the like, in order to discriminate between reactivity patterns of healthy control subjects to those of patients having brain injury. As such, this term specifically includes a difference measured by, for example, determining the reactivity of antibodies in a test sample to a plurality of antigens, and comparing the resulting reactivity pattern to the reactivity patterns of negative and positive control samples (e.g. samples obtained from control subjects which are not afflicted with brain injury or patients afflicted with brain injury, respectively) using such algorithms and/or analyzers. The difference may also be measured by comparing the reactivity pattern of the test sample to a predetermined classification rule obtained in such manner.

In some embodiments, the methods of the invention may employ the use of learning and pattern recognition analyzers, clustering algorithms and the like, in order to discriminate between reactivity patterns of subjects having a subtype of brain injury to control subjects. For example, the methods may include determining the reactivity of antibodies in a test sample to a plurality of antigens, and comparing the resulting pattern to the reactivity patterns of negative and positive control samples using such algorithms and/or analyzers.

Thus, in another embodiment, a significant difference between the reactivity patterns of a test sample compared to a reactivity pattern of a control sample, wherein the difference is computed using a learning and pattern recognition algorithm, indicates that the subject is afflicted with brain injury. For example, the algorithm may include, without limitation, supervised or non-supervised classifiers including statistical algorithms including, but not limited to, principal component analysis (PCA), partial least squares (PLS), multiple linear regression (MLR), principal component regression (PCR), discriminant function analysis (DFA) including linear discriminant analysis (LDA), and cluster analysis including nearest

neighbor, artificial neural networks, coupled two-way clustering algorithms, multi-layer perceptrons (MLP), generalized regression neural network (GRNN), fuzzy inference systems (FIS), self-organizing map (SOM), genetic algorithms (GAS), neuro-fuzzy systems (NFS), adaptive resonance theory (ART).

In certain embodiments, one or more algorithms or computer programs may be used for comparing the amount of each antibody quantified in the test sample against a predetermined cutoff (or against a number of predetermined cutoffs). Alternatively, one or more instructions for manually performing the necessary steps by a human can be provided.

Algorithms for determining and comparing pattern analysis include, but are not limited to, principal component analysis, Fischer linear analysis, neural network algorithms, genetic algorithms, fuzzy logic pattern recognition, and the like. After analysis is completed, the resulting information can, for example, be displayed on display, transmitted to a host computer, or stored on a storage device for subsequent retrieval.

Many of the algorithms are neural network based algorithms. A neural network has an input layer, processing layers and an output layer. The information in a neural network is distributed throughout the processing layers. The processing layers are made up of nodes that simulate the neurons by the interconnection to their nodes. Similar to statistical analysis revealing underlying patterns in a collection of data, neural networks locate consistent patterns in a collection of data, based on predetermined criteria.

Suitable pattern recognition algorithms include, but are not limited to, principal component analysis (PCA), Fisher linear discriminant analysis (FLDA), soft independent modeling of class analogy (SIMCA), K-nearest neighbors (KNN), neural networks, genetic algorithms, fuzzy logic, and other pattern recognition algorithms. In some embodiments, the Fisher linear discriminant analysis (FLDA) and canonical discriminant analysis (CDA) as well as combinations thereof are used to compare the output signature and the available data from the database.

In other embodiments, principal component analysis is used. Principal component analysis (PCA) involves a mathematical technique that transforms a number of correlated variables into a smaller number of uncorrelated variables. The smaller number of uncorrelated variables is known as principal components. The first principal component or eigenvector

accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible. The main objective of PCA is to reduce the dimensionality of the data set and to identify new underlying variables.

Principal component analysis compares the structure of two or more covariance matrices in a hierarchical fashion. For instance, one matrix might be identical to another except that each element of the matrix is multiplied by a single constant. The matrices are thus proportional to one another. More particularly, the matrices share identical eigenvectors (or principal components), but their eigenvalues differ by a constant. Another relationship between matrices is that they share principal components in common, but their eigenvalues differ. The mathematical technique used in principal component analysis is called eigenanalysis. The eigenvector associated with the largest eigenvalue has the same direction as the first principal component. The eigenvector associated with the second largest eigenvalue determines the direction of the second principal component. The sum of the eigenvalues equals the trace of the square matrix and the maximum number of eigenvectors equals the number of rows of this matrix.

In another embodiment, the algorithm is a classifier. One type of classifier is created by "training" the algorithm with data from the training set and whose performance is evaluated with the test set data. Examples of classifiers used in conjunction with the invention are discriminant analysis, decision tree analysis, receiver operator curves or split and score analysis.

The term “decision tree” refers to a classifier with a flow-chart-like tree structure employed for classification. Decision trees consist of repeated splits of a data set into subsets. Each split consists of a simple rule applied to one variable, e.g., “if value of “variable 1” larger than “threshold 1”; then go left, else go right”. Accordingly, the given feature space is partitioned into a set of rectangles with each rectangle assigned to one class.

The terms “test set” or “unknown” or “validation set” refer to a subset of the entire available data set consisting of those entries not included in the training set. Test data is applied to evaluate classifier performance.

The terms “training set” or “known set” or “reference set” refer to a subset of the respective entire available data set. This subset is typically randomly selected, and is solely used for the purpose of classifier construction.

Diagnostic methods

As used herein the term "diagnosing" or "diagnosis" refers to the process of identifying a medical condition or disease (e.g., brain injury) by its signs, symptoms, and in particular from the results of various diagnostic procedures, including e.g. detecting the reactivity, or reactivity pattern, of antibodies in a biological sample (e.g. serum) obtained from an individual, to one or more antigens. Furthermore, as used herein the term "diagnosing" or "diagnosis" encompasses screening for a disease, detecting a presence or a severity of a disease, distinguishing a disease from other diseases including those diseases that may feature one or more similar or identical symptoms, providing prognosis of a disease, monitoring disease progression or relapse, as well as assessment of treatment efficacy and/or relapse of a disease, disorder or condition, as well as selecting a therapy and/or a treatment for a disease, optimization of a given therapy (dose/schedule) for a disease, monitoring the treatment of a disease, and/or predicting the suitability of a therapy for specific patients or subpopulations or determining the appropriate dosing of a therapeutic product in patients or subpopulations.

In one embodiment, diagnosing brain injury further permits assessing a risk of said brain injury evolving to brain damage and leading to long-term dysfunction. In another embodiment, assessment of a risk of said brain injury evolving to long-term dysfunction permits therapeutic intervention at an early stage.

The immediate issue facing an individual that has suffered a TBI is determining when it is safe to return to high risk activities after a concussive injury without risking permanent brain damage that occurs at a cellular level. According to some embodiments, the present invention provides a broad immune system test to monitor, assess chronic outcomes and verify safety to return to work or play.

Assessment of pathology and neurological impairment immediately after TBI is crucial for determination of appropriate clinical management and for predicting long-term outcome. The outcome measures most often used in head injuries are the Glasgow Coma

Scale (GCS), the Glasgow Outcome Scale (GOS), computed tomography, and magnetic resonance imaging (MRI) to detect intracranial pathology. However, despite dramatically improved emergency triage systems based on these outcome measures, most TBI suffer long term impairment and a large number of TBI survivors are severely affected despite predictions of "good recovery" on the GOS. In addition, CT and MRI are expensive and cannot be rapidly employed in an emergency room environment. Moreover, in austere medical environments associated with combat, accurate diagnosis of TBI would be an essential prerequisite for appropriate triage of casualties.

In one embodiment, the type of brain damage associated with brain injury is a white matter structural abnormality. In another embodiment, the white matter structural abnormality or damage is in the corpus callosum region. In another embodiment, the abnormality or damage is in the uncinate fasciculus. In another embodiment, the abnormality or damage is in the right brain frontal lobe. In another embodiment, the abnormality or damage is in the left frontal lobe. In another embodiment, the abnormality or damage is diffuse axonal injury (DAI). In another embodiment, the abnormality or damage is diffuse vascular injury.

In some embodiments, the brain injury is a mild TBI, in one embodiment a concussion is a mild TBI. In another embodiment, mild TBI is caused by a head injury, where the head injury is, in another embodiment, blunt trauma, acceleration, or deceleration forces. It will be appreciated that such head injuries can be characterized by having one or more of the following conditions: (1) observed or self-reported contusion, disorientation, or impaired consciousness, dysfunction of memory at the time of the injury, loss of consciousness lasting less than 30 minutes; and, (2) symptoms such as headache, dizziness, fatigue, irritability, and poor concentration soon after the injury. Head injuries are also categorized as mild based on clinical examinations using the Glasgow Coma Scale. In one embodiment, the head injury has a Glasgow Coma Scale score (GCS) of 13-15 upon examination at an emergency center, with no abnormal findings on head CT, duration of loss of consciousness for no more than 30 minutes, post-traumatic amnesia for less than 24 hours, and an Abbreviated Injury Score (AIS) S3 and an ISS of <12 modified to exclude the head region.

Diagnostic methods differ in their sensitivity and specificity. The "sensitivity" of a diagnostic assay is the percentage of diseased individuals who test positive (percent of "true

positives"). Diseased individuals not detected by the assay are "false negatives." Subjects who are not diseased and who test negative in the assay, are termed "true negatives." The "specificity" of a diagnostic assay is 1 minus the false positive rate, where the "false positive" rate is defined as the proportion of those without the disease who test positive. While a particular diagnostic method may not provide a definitive diagnosis of a condition, it suffices if the method provides a positive indication that aids in diagnosis. The "accuracy" of a diagnostic assay is the proximity of measurement results to the true value. The "p value" of a diagnostic assay is the probability of obtaining the observed sample results (or a more extreme result) when the null hypothesis is actually true.

In certain embodiments, the use of an antigen probe set provided by the present invention, or an antigen probe array provided by the present invention, results in an antibody reactivity profile which is brain injury-indicative (p value $\leq$ 1.00E-08), sensitive ($\geq$ 0.600), specific ($\geq$ 0.700) and accurate ($\geq$ 0.600). In certain embodiments, the use results in an antibody reactivity profile which is more brain injury-indicative (p value $\leq$ 1.00E-10), sensitive ($\geq$ 0.700), specific ($\geq$ 0.800) and accurate ($\geq$ 0.700). In certain embodiments, the use results in an antibody reactivity profile which is even more brain injury-indicative (p value $\leq$ 1.00E-12), sensitive ($\geq$ 0.800), specific ($\geq$ 0.900) and accurate ($\geq$ 0.800). In certain embodiments, the use results in an antibody reactivity profile which is yet even more brain injury -indicative (p value $\leq$ 1.00E-14), sensitive ($\geq$ 0.900), specific ($\geq$ 0.950) and accurate ($\geq$ 0.900). In certain embodiments, the use results in an antibody reactivity profile which highly brain injury -indicative (p value $\leq$ 1.00E-16), sensitive ($\geq$ 0.950), specific ($\geq$ 0.990) and accurate ($\geq$ 0.950). Each possibility represents a separate embodiment of the invention.

In certain embodiments, the antigens provided by the present invention, or the antigen patterns provided by the present invention, are brain injury -indicative (p value $\leq$ 1.87E-08), sensitive ($\geq$ 0.609), specific ($\geq$ 0.769) and accurate ($\geq$ 0.687). In certain embodiments, the antigens provided by the present invention, or the antigen patterns provided by the present invention, are advantageously brain injury-indicative (p value $\leq$ 2.81E-12), sensitive ($\geq$ 0.657), specific ($\geq$ 0.798) and accurate ($\geq$ 0.725). In certain embodiments, the antigens provided by the present invention, or the antigen patterns provided by the present invention, are further advantageously brain injury-indicative (p value $\leq$ 8.00E-14), sensitive ($\geq$ 0.663), specific ($\geq$ 0.814) and accurate ($\geq$ 0.738).

In another embodiment, the methods may result in determining a level of brain injury progression. In a further embodiment, the methods may result in providing the comparison to an entity for monitoring brain injury progression. In these embodiments, the methods can be used, for example, to differentiate between subjects with progressing brain injury, and subjects with regressing brain injury.

In one embodiment, the subject being diagnosed according to the methods of the invention is symptomatic. In other embodiments, the subject is asymptomatic. In certain embodiments the subject shows immediate symptoms. In certain embodiments the subject shows delayed symptoms. In certain embodiments, the subject is not or was not receiving a treatment.

As used herein, the term "treating" may encompass curing, preventing, reducing the incidence of, ameliorating symptoms of, to inducing remission of, or slowing the progression of a disease. The terms "reducing", "suppressing" and "inhibiting" refer to lessening or decreasing.

The diagnostic procedure can be performed in vivo or in vitro, preferably in vitro. In certain embodiments of the methods of the present invention, the diagnostic procedure is performed by non-invasive means or methods.

The diagnostic procedure and platform of the present invention may be suitable for use as point of care device or point of service in clinic, in physician's office, in hospital laboratories, or in commercial diagnostic laboratories.

The following examples are presented in order to more fully illustrate some embodiments of the invention. They should, in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLES

Materials and Methods

Human subjects

The study was approved by the Institutional Review Boards of the participating clinical unit; informed consent was obtained from all participants. In an initial study, sera

derived from blood samples obtained from healthy subjects, and subjects suffering from brain injury at varying times post injury, and with varying GOSE scores, were tested using an antigen microarray that included 228 antigens (see Table 1).

Blood samples and clinical data were collected from patients in the HeadSMART trial, arriving at the emergency departments (ED) of Johns Hopkins Hospital (JHH, Baltimore; n= 61) or at one of the participating centers of the COBRIT clinical trial (n= 31; as described in JAMA. 2012;308(19):1993-2000).

Defined human serum samples were used for this study. Samples from adult TBI patients were analyzed retrospectively. The healthy control cohort of patients, evaluated for non-TBI complaints was obtained from Baylor College of Medicine (Houston, TX; n= 21).

To be considered a TBI patient for the HeadSMART trial, the following criteria had to be met: 18 years old or greater, blunt TBI presenting within 24 hours of injury, met the American College of Emergency Physicians (ACEP) criteria for obtaining head CT scans in TBI. Patients having brain tumor, brain surgery, pregnant, non-English speakers, were excluded. Serial serum samples were collected from enrollment to up to 6 months from 61 TBI patients. Three samples per patient at eight different time points after brain injury were collected, a selection of which were used in the analysis For COBRIT trial samples, the following criteria were used: Inclusion Criteria were that the patient had a non-penetrating traumatic brain injury, age 18 (19 in Alabama) - 70 years, GCS criteria on/off paralytics as specified in protocol, reasonable expectation of completion of outcome measures at a network center at six months post-injury, reasonable expectation of enrollment within 24-hour time window, and English-speaking. Exclusion criteria included: Intubated patients with GCS motor score = 6 and not meeting CT criteria, bilaterally fixed and dilated pupils, positive pregnancy test, known pregnancy, or currently breast feeding, evidence of diseases that interfere with outcome assessment, current acetylcholinesterase inhibitor use, imminent death or current life-threatening disease, currently enrolment in another study, or prisoners. For healthy controls, 21 non-TBI individuals at least 18 years of age were recruited under informed consent at Baylor College of Medicine. One blood sample was collected per control individual and processed to obtain replicate vials of serum and plasma, which were stored at -80° Celsius until use. All patient identifiers were kept confidential.

Antigens and serum testing

228 different antigens were spotted on in-house produced epoxyhexyltriethoxysilane (EHTES) activated epoxy slides using a Scienion S-11 non-contact microarray printer (Scienion AG, Germany). The microarrays were then blocked for 1 hour at room temperature with 1% casein. Test serum samples in 1% casein blocking buffer (1:20 dilution) were incubated under a coverslip for 1 hour at 37°. The arrays were then washed and incubated for 1 hour at 37° with a 1:500 dilution of two detection antibodies, mixed together: a goat anti-human IgG Cy3-conjugated antibody, and a goat anti-human IgM AF647-conjugated antibody (both purchased from Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Image acquisition was performed by laser at two wavelengths 530nm and 630 nm (Agilent Technologies, Santa Clara, CA) and the results were analyzed using Genepix pro 7 software (Molecular devices, Sunnyvale, CA). The quantitative range of signal intensity of binding to each antigen spot was 0–65,000; this range of detection made it possible to obtain reliable data at a 1:20 dilution of test serum samples.

Image analysis and data processing

Each spot's intensity is represented by its pixels' mean after subtraction of its local background median, followed by Log2 transform. Negative spots (following background subtraction) are imputed with background-like intensity. Background intensity was subtracted for each spot, to obtain net signals. For every antigen in every slide, outlier spots were removed. Outliers spots are defined as having Z score >2 or <-2. The intensity of multiple spots was combined through median, following removal of outlier spots. The foreground and background intensities of multiple spots of each antigen were averaged, and the difference between the foreground and the background was calculated. The resulting value was taken as the antigen reactivity of the antibodies binding to that spotted antigen. All antigens showed meaningful reactivity in a significant number of slides; thus no antigen was excluded.

Statistical analysis of antibody results

Antigens whose reactivity was higher or lower in a specific study subgroup compared to other subgroups were identified. Univariare analysis was used for separating antigens in a T test. Antigens that allowed for setting a classification threshold such as positive

predictive value (PPV) ≥ 90% and sensitivity ≥ 20% were achieved and determined to significantly characterize a specific subgroup. For added restriction, only antigens whose p value for a two sided t-test (after Benjamini–Hochberg correction for multiple hypothesis) was smaller than 0.05 were selected.

ELISA plate assay methods

Biomarkers are tested by either the colorimetric, fluorescence, chemiluminescent, or electrochemiluminescent detection methodologies. For the colorimetric detection methods, Maxisorb 96 well plates are used. For fluorescence assays, black opaque-walled plates are used. For luminescence based-assays, microtiter plates suitable for luminescence are used. Plates are prepared as follows. Plates are rinsed once with coating buffer specific to each plate type. Capture antibodies are added to each well at an optimized concentration for an optimal time period. Generally coating is performed over 12 hours at 4^0 C in optimal coating buffer. Following the coating period, the excess antibody is removed and the plates are blocked in an optimized blocking buffer consisting of buffered saline with one of the following: Casein, bovine serum Albumin, species-specific whole serum, or filtered non-fat dry milk powder, or other blocking agent, and/or non-ionic detergent. A series of sequential incubations of optimal length are used to allow: 1) masking of non-specific binding sites (i.e., blocking), 2), capture antibody-antigen binding, 3) binding of antigen followed by washing for removal of excess and non-bound antigens, 4) incubation of anti-antigen detection antibody solution and detection tag, 5) washing for removal of excess non-bound detection antibody and tag, and 6) addition of detection substrate (ELISA) or optimal detection solution (fluorescence or luminescence). Colorimetric detection is performed on a microtiter plate reader, or similar technology, by measuring absorbance of a colored substrate at an appropriate wavelength of light. Fluorescence assays are performed using a fluorescence based plate reader. Luminescence is detected on a luminescence based reader. Data are collected and biomarker concentrations are determined using a standard curve of recombinant protein of known concentration.

EXAMPLE 1: Association between FABP (SEQ ID No: 61) and MBPR149 (SEQ ID No: 10) with TBI outcomes

Each patient was profiled with its own measured time-points in order to explore its autoantibodies profile change with time post injury. Samples from TBI patients with

Extended Glasgow Outcome Scale (GOSE) equals 8 were compared to samples from TBI patients with GOSE lower than 8 at a specific time-point (3 months/ 1 month).

Antibodies' binding

Sera samples from healthy subjects and brain injury patients at varying times post injury, and with varying GOSE scores were tested for binding of serum IgG and/or IgM antibodies to the various antigens disclosed in Table 1.

Table 1: List of brain injury related antigens.

Antigen	Amino acid sequence or manufacture (Catalog number)	SEQ ID NO:
MBP (myelin basic protein)	MASQKRPSQRHGSKYLATASTMDHARHGFLPRHRDTGILDSIG RFFGGDRGAPKRGSGKVPWLKPGRSPL PSHARSQPGLCNMYKDSHHPARTAHYGSLPQKSHGRTQDENP VVHFFKNIVTPRTPPPSQGKGRGLSLSR FSWGAEGQRPGFGYGGRASDYKSAHKGFKGVDAQGTLSKIFK LGGRDSRSGSPMARR Enzo LS (ALX-200-606-M001)	1
MBP- in vitro citrullinated	Post translational citullination of arginine(s) in Enzo LS (ALX-200-606-M001)	2
MBP R26	Ac-TMDHA(Cit)HGFLPC-amide	3
MBP R32, R34	Ac-GFLP(Cit)H(Cit)DTGIC-amide	4
MBP R44	Ac-CILDSIG(Cit)FFGG-amide	5
MBP R50	Ac-FGGD(Cit)GAPKRGC-amide	6
MBP R92	Ac-CDSHHPA(Cit)TAHYG-amide	7
MBP R106	Ac-CQKSHG(Cit)TQDEN-amide	8
MBP R124	Ac-CFKNIVTP(Cit)TP-amide	9
MBP R149	Ac-GAEGQ(Cit)PGFGYC-amide	10
MBP R157	Ac-CGYGG(Cit)ASDYKS-amide	11
MBP R186,	Ac-CKLGG(Cit)DS(Cit)SG-amide	12

R189		
MBP R196, R197	Ac-C(Ahx)SGSPMA(Cit)(Cit)-OH	13
GFAP (glial fibrillary acid protein)	MERRRITSAARRSYVSSGEMMVGGLAPGRRLGPGTRLSLARM PPPLPTRVDFSLAGALNAGFKETRASER AEMMELNDRFASYIEKVRFLEQQNKALAAELNQLRAKEPTKL ADVYQAELRELRLRLDQLTANSARLEVE RDNLAQDLATVRQKLQDETNLRLEAENNLAAYRQEADEATL ARLDLERKIESLEEEIRFLRKIHEEEVRE LQEQLARQQVHVELDVAKPDLTAALKEIRTQYEAMASSNMHE AEEWYRSKFADLTDAAARNAELLRQAKH EANDYRRQLQSLTCDLESLRGTNESLERQMREQEERHVREAA SYQEALARLEEEGQSLKDEMARHLQEYQ DLLNVKLALDIEIATYRKLLEGEENRITIPVQTFSNLQIRETSLD TKSVSEGHLKRNIVVKTVEMRDGEV IKESKQEHKDVM Calbiochem (345996)	14
GFAP- in vitro citrullinated	Post translational citullination of arginine(s) in Calbiochem (345996)	15
GFAP R30	Ac-LAPGR(Cit)LGPGTC-amide	16
GFAP R36	Ac-CLGPGT(Cit)LSLAR-amide	17
GFAP R270	Ac-AA(Cit)NAELLRQC-amide	18
GFAP R406	Ac-CEGHLK(Cit)NIVVK-amide	19
GFAP R416	Ac-CVKTVEM(Cit)DGEVI-amide	20
NRGN (neurogranin)	MDCCTENACSKPDDDILDIPLDDPGANAAAAKIQASFRGHMA RKKIKSGERGRKGPGPGGPGGAGVARGG AGGGPSGD	21
NRGN- in vitro citrullinated	Post translational citullination of arginine(s) in NRGN	22
NRGN R51, R53	Ac-CKSGE(Cit)G(Cit)KGPG-amide	23
NRGN R68	Ac-CGGAGVA(Cit)GGAG-amide	24
ERMIN	MKTLSPDRIQPHIMTDVPATFTQAECNGDKPPENGQQTITKISE ELTDVDSPLPHYRVEPSLEGALTKGS QEERRKLQGNMLLNSSMEDKMLKENPEEKLFIVHKAITDLSLQ ETSADEMTFREGHQWEKIPLSGSNQEI RRQKERITEQPLKEEEDEDRKNKGHQAAEIEWLGFRKPSQAD MLHSKHDEEQKVWDEEIDDDDDDNCNND	25

	EDEVRVIEFKKKHEEVSQFKEEGDASEDSPLSSASSQAVTPDEQ PTLGKKSDISRNAYSRYNTISYRKIR KGNTKQRIDEFESMMHL	
ERMIN-in vitro citrullinated	Post translational citullination of arginine(s) in ERMIN	26
Ermin R57	Ac-DSPLPHY(Cit)VEPSLEC-amide	27
ICAM5	MPGPSPGLRRALLGLWAALGLGLFGLSAVSQEPFWADLQPRV AFVERGGSLWLNCSTNCPRPERGGLETS LRRNGTQRGLRWLARQLVDIREPETQPVCFFRCARRTLQARGL IRTFQRPDRVELMPLPPWQPVGENFTL SCRVPGAGPRASLTLTLLRGAQELIRRSFAGEPPRARGAVLTAT VLARREDHGANFSCRAELDLRPHGLG LFENSSAPRELRTFSLSPDAPRLAAPRLLEVGSERPVSCTLDGLF PASEARVYLALGDQNLSPDVTLEGD AFVATATATASAEQEGARQLVCNVTLGGENRETRENVTIYSFP APLLTLSEPSVSEGQMVTVTCAAGAQA LVTLEGVPAAVPGQPAQLQLNATENDDRRSFFCDATLDVDGE TLIKNRSAELRVLYAPRLDDSDCPRSWT WPEGPEQTLRCEARGNPEPSVHCARSDGGAVLALGLLGPVTR ALSGTYRCKAANDQGEAVKDVTLTVEYA PALDSVGCPERITWLEGTEASLSCVAHGVPPPDVICVRSGELGA VIEGLLRVAREHAGTYRCEATNPRGS AAKNVAVTVEYGPRFEEPSCPSNWTWVEGSGRLFSCEVDGKP QPSVKCVGSGGATEGVLLPLAPPDPSPR APRIPRVLAPGIYVCNATNRHGSVAKTVVVSAESPPEMDESTC PSHQTWLEGAEASALACAARGRPSPGV RCSREGIPWPEQQRVSREDAGTYHCVATNAHGTDSRTVTVGV EYRPVVAELAASPPGGVRPGGNFTLTCR AEAWPPAQISWRAPPGALNIGLSSNNSTLSVAGAMGSHGGEYE CAATNAHGRHARRITVRVAGPWLWVAV GGAAGGAALLAAGAGLAFYVQSTACKKGEYNVQEAESSGEA VCLNGAGGGAGGAAGAEGGPEAAGGAAES PAEGEVFAIQLTSA R&D (1950-M5)	28
SNCB (Beta-synuclein)	MDVFMKGLSMAKEGVVAAAEKTKQGVTEAAEKTKEGVLYV GSKTREGVVQGVASVAEKTKEQASHLGGAV FSGAGNIAAATGLVKREEFPTDLKPEEVAQEAAEEPLIEPLMEP EGESYEDPPQEEYQEYEPEA OriGene (TP315165)	29
MT3 (Metallothionein III)	MDPETCPCPSGGSCTCADSCKCEGCKCTSCKKSCCSCCPAECEK CAKDCVCKGGEAAEAEAEKCSCCQ	30
OMG (Oligodenrocyte	MEYQILKMSLCLFILLFLTPGILCICPLQCICTERHRHVDCSGRN LSTLPSGLQENIIHLNLSYNHFTDL HNQLTQYTNLRTLDISNNRLESLPAHLPRSLWNMSAANNNIKL	31

Myelin Glycoprotein)	LDKSDTAYQWNLKYLDVSKNMLEKVVL IKNTLRSLEVLNLSSNKLWTVPTNMPSKLHIVDLSNNSLTQILP GTLINLTNLTHLYLHNNKFTFIPDQS FDQLFQLQEITLYNNRWSCDHKQNITYLLKWMMETKAHVIGT PCSTQISSLKEHNMYPTPSGFTSSLFTV SGMQTVDTINSLSVVTQPKVTKIPKQYRTKETTFGATLSKDTTF TSTDKAFVPYPEDTSTETINSHEAAA ATLTIHLQDGMVTNTSLTSSTKSSPTPMTLSITSGMPNNFSEMP QQSTTLNLWREETTTNVKTPLPSVAN AWKVNASFLLLLNVVVMLAV	
CNDP1 (Carnosine dipeptidase 1)		32
Reticulon 1	MAAPGDPQDELLPLAGPGSQWLRHRGEGENEAVTPKGATPAP QAGEPSPGLGARAREAASREAGSGPARQ SPVAMETASTGVAGVSSAMDHTFSTTSKDGEGSCYTSLISDICY PPQEDSTYFTGILQKENGHVTISESP EELGTPGPSLPDVPGIESRGLFSSDSGIEMTPAESTEVNKILADP LDQMKAEAYKYIDITRPEEVKHQEQ HHPELEDKDLDFKNKDTDISIKPEGVREPDKPAPVEGKIIKDHL LEESTFAPYIDDLSEEQRRAPQITTP VKITLTEIEPSVETTTQEKTPEKQDICLKPSPDTVPTVTVSEPED DSPGSITPPSSGTEPSAAESQGKGS ISEDELITAIKEAKGLSYETAENPRPVGQLADRPEVKARSGPPTI PSPLDHEASSAESGDSEIELVSEDP MAAEDALPSGYVSFGHVGGPPPSPASPSIQYSILREEREAELDS ELIIESCDASSASEESPKREQDSPPM KPSALDAIREETGVRAEERAPSRRGLAEPGSFLDYPSTEPQPGP ELPPGDGALEPETPMLPRKPEEDSSS NQSPAATKGPGPLGPGAPPPLLFLNKQKAIDLLYWRDIKQTGIV FGSFLLLLFSLTQFSVVSVVAYLALA ALSATISFRIYKSVLQAVQKTDEGHPFKAYLELEITLSQEQIQKY TDCLQFYVNSTLKELRRLFLVQDLV DSLKFAVLMWLLTYVGALFNGLTLLLMAVVSMFTLPVVYVK HQAQIDQYLGLVRTHINAVVAKIQAKIPG AKRHAE	33
Astrotactin 1	MALAGLCALLACCWGPAAVLATAAGDVDPSKELECKLKSITV SALPFLRENDLSIMHSPSASEPKLLFSV RNDFPGEMVVVDDLENTELPYFVLEISGNTEDIPLVRWRQQWL ENGTLLFHIHHQDGAPSLPGQDPTEEP QHESAEEELRILHISVMGGMIALLLSILCLVMILYTRRRWCKRR RVPQPQKSASAEAANEIHYIPSVLIG GHGRESLRNARVQGHNSSGTLSIRETPILDGYEYDITDLRHHLQ RECMNGGEDFASQVTRTLDSLQGCNE KSGMDLTPGSDNAKLSLMNKYKDNIIATSPVDSNHQQATLLSH TSSSQRKRINNKARAGSAFLNPEGDSG TEAENDPQLTFYTDPSRSRRRSRVGSPRSPVNKTTLTLISITSCVI GLVCSSHVNCPLVVKITLHVPEHL	34

	IADGSRFILLEGSQLDASDWLNPAQVVLFSQQNSSGPWAMDLC ARRLLDPCEHQCDPETGECLCYEGYMK DPVHKHLCIRNEWGTNQGPWPYTIFQRGFDLVLGEQPSDKIFR FTYTLGEGMWLPLSKSFVIPPAELAIN PSAKCKTDMTVMEDAVEVREELMTSSSFDSLEVLLDSFGPVRD CSKDNGGCSKNFRCISDRKLDSTGCVC PSGLSPMKDSSGCYDRHIGVDCSDGFNGGCEQLCLQQMAPFP DDPTLYNILMFCGCIEDYKLGVDGRSCQ LITETCPEGSDCGESRELPMNQTLFGEMFFGYNNHSKEVAAGQ VLKGTFRQNNFARGLDQQLPDGLVVAT VPLENQCLEEISEPTPDPDFLTGMVNFSEVSGYPVLQHWKVRS VMYHIKLNQVAISQALSNALHSLDGAT SRADFVALLDQFGNHYIQEAIYGFEESCSIWYPNKQVQRRLWL EYEDISKGNSPSDESEERERDPKVLTF PEYITSLSDSGTKHMAAGVRMECHSKGRCPSSCPLCHVTSSPD TPAEPVLLEVTKAAPIYELVTNNQTQR LLQEATMSSLWCSGTGDVIEDWCRCDSTAFGADGLPTCAPLP QPVLRLSTVHEPSSTLVVLEWEHSEPPI GVQIVDYLLRQEKVTDRMDHSKVETETVLSFVDDIISGAKSPC AMPSQVPDKQLTTISLIIRCLEPDTIY MFTLWGVDNTGRRSRPSDVIVKTPCPVVDDVKAQEIADKIYNL FNGYTSGKEQQTAYNTLLDLGSPTLHR VLYHYNQHYESFGEFTWRCEDELGPRKAGLILSQLGDLSSWC NGLLQEPKISLRRSSLKYLGCRYSEIKP YGLDWAELSRDLRKTCEEQTLSIPYNDYGDSKEI	
Brain Angiogenesis Inhibitor 3	uniprot# O60242	35
Glutamate Receptor, Metabotrophic 3	uniprot# Q14832	36
Kelch like 32	uniprot# Q96NJ5	37
Matrix metalloproteinase-9	uniprot# P14780	38
Melanoma Antigen Family E, 2	uniprot# Q8TD90	39
Neuregulin 3	uniprot# P56975	40
SLIT and NTRK-Like Family,	uniprot#O94933	41

Member 3		
BDNF (Brain derived neurotrophic factor)	MTILFLTMVISYFGCMKAAPMKEANIRGQGGLAYPGVRTHGT LESVNGPKAGSRGLTSLADTFEHVIEEL LDEDQKVRPNEENNKDADLYTSRVMLSSQVPLEPPLLFLLEEY KNYLDAANMSMRVRRHSDPARRGELSV CDSISEWVTAADKKTAVDMSGGTVTVLEKVPVSKGQLKQYFY ETKCNPMGYTKEGCRGIDKRHWNSQCRT TQSYVRALTMDSKKRIGWRFIRIDTSCVCTLTIKRGR R&D (248BD005)	42
UBIQUITIN CTERMINAL HYDROLASE L1	uniprot# P09936	43
Oligo24	T16G1: TTT TTT TTT TTT TTT TG	44
Tubulin beta-4B chain in vitro citrullinated	uniprot# P68371	45
Tubulin beta-4B chain	(K)IREEYPDrIMNTF(S)	46
Tubulin alpha-1B chain	uniprot# P68363	47
Tubulin alpha-1B chain in vitro citrullinated		48
Tubulin alpha-1B chain	(K)YMAccLLYrGDVVPK(D)	49
Tubulin alpha-1B chain	(E)VrTGTYrQLFHPE(Q)	50
synaptotagmin	uniprot# P21579	51
AB1-42		52
CNPase	(K)STLArVIVDK(Y)	53
CNPase	(K)ITPGArGAFSEEYK(R)	54
Laminin	uniprot# Q13753	55
PPIA in	uniprot# P62937	56

vitro citrullinated		
PPIA	(K)TAENFrALSTGEK(G)	57
S100A10	Uniprot# P60903	58
Septin-7 in vitro citrullinated		59
Septin-7	(R)ILEQQNSSrTLEK(N)	60
Fatty acid binding protein (FABP-3)	Prospec (PRO-340)	61
Elongation factor 1-alpha 2 in vitro citrullinated	Uniprot#Q05639	62
Elongation factor 1-alpha 2	(K)PLrLPLQDVYK(I)	63
Elongation factor 1-alpha 2	(D)VYKIGGIGTVPVGrVE(T)	64
ICNPase (2',3'-cyclic nucleotide 3'-phosphodiesterase)	uniprot# P09543	65
Collagen-IV	uniprot# P02462	66
TPPP	(K)AISSPTVSrLTDTTK(F)	67
Phospho-c-Jun	uniprot# P05412	68
TPPP3 in vitro citrullinated	(K)TGGAVD(Cit)LTDTSrYTGSHK(E)	69
TPPP3	(K)TGGAVDRLTDTSrYTGSHK(E)	70
TPPP3	(K)GIAGrQDILDDSGYVSAYK(N)	71
vesicular membrane protein	uniprot# Q8IZ57	72

neurensin-1 (p24		
NDRG2, Isoform 2 in vitro citrullinated	uniprot# Q9UN36	73
NDRG2, Isoform 2	(R)TASLTSAASVDGNrSR(S)	74
S100 calcium binding protein B (S100B)	MSELEKAMVALIDVFHQYSGREGDKHKLKKSELKELINNELSH FLEEIKEQEVVDKVMETLDNDGDGECD FQEFMAFVAMVTTACHEFFEHE Sigma (S6677)	75
NSE (neuron specific enolase aka ENO2)	MSIEKIWAREILDSRGNPTVEVDLYTAKGLFRAAVPSGASTGIY EALELRDGDKQRYLGKGVLKAVDHIN STIAPALISSGLSVVEQEKLDNLMLELDGTENKSKFGANAILGV SLAVCKAGAAERELPLYRHIAQLAGN SDLILPVPAFNVINGGSHAGNKLAMQEFMILPVGAESFRDAMR LGAEVYHTLKGVIKDKYGKDATNVGDE GGFAPNILENSEALELVKEAIDKAGYTEKIVIGMDVAASEFYRD GKYDLDFKSPTDPSRYITGDQLGALY QDFVRDYPVVSIEDPFDQDDWAAWSKFTANVGIQIVGDDLTV TNPKRIERAVEEKACNCLLLKVNQIGSV TEAIQACKLAQENGWGVMVSHRSGETEDTFIADLVVGLCTGQI KTGAPCRSERLAKYNQLMRIEEELGDE ARFAGHNFRNPSVL Abnova (H00002026-P01)	76
MCP1 (monocyte chemotactic protein-1)	Prospec (CHM-271)	77
Tau, total	Sigma (T9392)	78
Neurofilament light polypeptide		79
Neurofilament heavy polypeptide		80
y-Enolase		81
Prothrombin-FactorII		82
EXOSC10	uniprot# Q01780	83
Spectrin,	Sigma (S3644)	84

breakdown products		
Myeloperoxidase (MPO)	Sigma (M6908)	85
CMV	Prospec (CMV Pp150)	86
ICAM	uniprot# Q8N6I2	87
SLC39A11	uniprot# Q8N1S5	88
MAP2 (Microtubule-associated protein 2		89
MAPT (microtubule-associated protein tau gene)		90
HTR1A (Serotonin receptor 1A gene)		91
PLXNA4 (PlexinsA4)		92
Interleukin-6	PVPPGEDSKD VAAPHRQPLT SSERIDKQIR YILDGISALR KETCNKSNMC ESSKEALAEN NLNLPKMAEK DGCFQSGFNE ETCLVKIITG LLEFEVYLEY LQNRFESSEE QARAVQMSTK VLIQFLQKKA KNLDAITTPD PTTNASLLTK LQAQNQWLQD MTTHLILRSF KEFLQSSLRA LRQM Peprotech (200-06)	93
Interleukin-12	p40 Subunit: IWELKK DVYVVELDWY PDAPGEMVVL TCDTPEEDGI TWTLDQSSEV LGSGKTLTIQ VKEFGDAGQY TCHKGGEVLS HSLLLLHKKE DGIWSTDILK DQKEPKNKTF LRCEAKNYSG RFTCWWLTTI STDLTFSVKS SRGSSDPQGV TCGAATLSAE RVRGDNKEYE YSVECQEDSA CPAAEESLPI EVMVDAVHKL KYENYTSSFF IRDIIKPDPP KNLQLKPLKN SRQVEVSWEY PDTWSTPHSY FSLTFCVQVQ GKSKREKKDR VFTDKTSATV ICRKNASISV RAQDRYYSSS WSEWASVPCS Peprotech (200-12)	94
Interleukin-15	MNWVNVISDL KKIEDLIQSM HIDATLYTES DVHPSCKVTA MKCFLLELQV ISLESGDASI HDTVENLIIL ANNSLSSNGN VTESGCKECE ELEEKNIKEF LQSFVHIVQM FINTS Peprotech (200-15)	95

Interleukin -17	MIVKAGITIP RNPGCPNSED KNFPRTVMVN LNIHNRNTNT NPKRSSDYYN RSTSPWNLHR NEDPERYPSV IWEAKCRHLG CINADGNVDY HMNSVPIQQE ILVLRREPPH CPNSFRLEKI LVSVGCTCVT PIVHHVA Peprotech (200-17)	96
Interleukin -1ra	MRPSGRKSSK MQAFRIWDVN QKTFYLRNNQ LVAGYLQGPN VNLEEKIDVV PIEPHALFLG IHGGKMCLSC VKSGDETRLQ LEAVNITDLS ENRKQDKRFA FIRSDSGPTT SFESAACPGW FLCTAMEADQ PVSLTNMPDE GVMVTKFYFQ EDE Peprotech (200-01RA)	97
TNFRI	MDSVCPQGKY IHPQNNSICC TKCHKGTYLY NDCPGPGQDT DCRECESGSF TASENHLRHC LSCSKCRKEM GQVEISSCTV DRDTVCGCRK NQYRHYWSEN LFQCFNCSLC LNGTVHLSCQ EKQNTVCTCH AGFFLRENEC VSCSNCKKSL ECTKLCLPQI EN Peprotech (310-07)	98
VEGF	APMAEGGGQN HHEVVKFMDV YQRSYCHPIE TLVDIFQEYP DEIEYIFKPS CVPLMRCGGC CNDEGLECVP TEESNITMQI MRIKPHQGQH IGEMSFLQHN KCECRPKKDR ARQENPCGPC SERRKHLFVQ DPQTCKCSCK NTDSRCKARQ LELNERTCRC DKPRR Peprotech (100-20)	99
VCAM1	FKIETTPESR YLAQIGDSVS LTCSTTGCES PFFSWRTQID SPLNGKVTNE GTTSTLTMNP VSFGNEHSYL CTATCESRKL EKGIQVEIYS FPKDPEIHLS GPLEAGKPIT VKCSVADVYP FDRLEIDLLK GDHLMKSQEF LEDADRKSLE TKSLEVTFTP VIEDIGKVLV CRAKLHIDEM DSVPTVRQAV KELQVYISPK NTVISVNPST KLQEGGSVTM TCSSEGLPAP EIFWSKKLDN GNLQHLSGNA TLTLIAMRME DSGIYVCEGV NLIGKNRKEV ELIVQEKPFT VEISPGPRIA AQIGDSVMLT CSVMGCESPS FSWRTQIDSP LSGKVRSEGT NSTLTLSPVS FENEHSYLCT VTCGHKKLEK GIQVELYSFP RDPEIEMSGG LVNGSSVTVS CKVPSVYPLD RLEIELLKGE TILENIEFLE DTDMKSLENK SLEMTFIPTI EDTGKALVCQ AKLHIDDMEF EPKQRQSTQT LYVNVAPRDT TVLVSPSSIL EEGSSVNMTC LSQGFPAPKI LWSRQLPNGE LQPLSENATL TLISTKMEDS GVYLCEGINQ AGRSRKEVEL IIQVTPKDIK LTAFPSESVK EGDTVIISCT CGNVPETWII LKKKAETGDT VLKSIDGAYT IRKAQLKDAG VYECESKNKV GSQLRSLTLD VQGRENNKDY FSP Peprotech (150-04)	100
FactorVIIa	AKRONbiotech (AK9916)	101
Collagen II		102
Microglobulin-b2	Sigma (M4890)	103
TNFRSF12A	EQAPGTAPCS RGSSWSADLD KCMDCASCRA RPHSDFCLGC AAAPPAPFRL LWP Peprotech (310-21)	104

TNFRII	MAPEPGSTCR LREYYDQTAQ MCCSKCSPGQ HAKVFCTKTS DTVCDSCEDS TYTQLWNWVP ECLSCGSRCS SDQVETQACT REQNRICTCR PGWYCALSKQ EGCRLCAPLR KCRPGFGVAR PGTETSDVVC KPCAPGTFSN TTSSTDICRP HQICNVVAIP GNASMDAVCT STSP Peprotech (310-12)	105
CRP	Sigma (C4063)	106
BAFF-R	MRRGPRSLRG RDAPAPTPCV PAECFDLLVR HCVACGLLRT PRPKPAGASS PAPRTALQPQ ESVGAGAGEA ALPLPG Peprotech (310-13R)	107
BAFF	AVQGPEETVT QDCLQLIADS ETPTIQKGSY TFVPWLLSFK RGSALEEKEN KILVKETGYF FIYGQVLYTD KTYAMGHLIQ RKKVHVFGDE LSLVTLFRCI QNMPETLPNN SCYSAGIAKL EEGDELQLAI PRENAQISLD GDVTFFGALK LL Peprotech (310-13)	108
GLP1	HAEGTFTSDV SSYLEGQAAK EFIAWLVKGR G Peprotech (130-08)	109
HSP90	Sigma (H6774)	110
EGFP	Prospec (cyt-332)	111
C4	Sigma (C8195)	112
C3	Sigma (C2910)	113
C1q	Prospec (pro-554)	114
Fibrinogen	AKRONbiotech (AK9026)	115

As shown in Figure 1, the levels of anti- Fatty acid-binding protein (FABP, SEQ ID No: 61) IgM autoantibodies in serum samples obtained from TBI patients at day 30 post injury, with Glasgow Outcome Scale Extended (GOSE) score <8 (cross labeled) are lower in comparison to patients with GOSE score=8 (circle labeled).

As shown in Figure 2, the levels of anti-Myelin basic protein (MBPR149, SEQ ID No: 10, MBP derived BSA conjugated peptide) IgM autoantibodies in serum samples obtained from TBI patients at day 30 post injury, with Glasgow Outcome Scale Extended (GOSE) score <8 (cross labeled) are higher in comparison to patients with GOSE score=8 (circle labeled). These results demonstrate for the first time that increased levels of anti-FABP IgM autoantibodies in serum samples obtained from a TBI patient are indicative of

recovery from brain injury of said TBI patient. Furthermore, decreased levels of anti-MBPR149 IgM autoantibodies in serum samples obtained from a TBI patient are indicative of recovery from brain injury of said TBI patient. Thus the present invention disclosed specific antigen antibody reactivities that can be used for monitoring, and/or prognosis of brain injury.

EXAMPLE 2: Elevated levels of Anti-Myeloperoxidase (MPO, SEQ ID No: 85) IgM autoantibodies in serum samples obtained from TBI patients as compared to healthy controls

As shown in Figure 3, the levels of anti-MPO IgM autoantibodies in serum samples obtained from TBI patients (circle labeled) are higher in comparison with healthy controls (cross labeled). These results demonstrate for the first time that increased levels of anti-MPO autoantibodies are indicative of brain injury.

EXAMPLE 3: Decreased levels of Anti- CMV (SEQ ID No: 86) IgG autoantibody in serum samples obtained from TBI patients as compared to healthy controls

As shown in Figure 4A, the levels of anti-CMV (SEQ ID No: 86) IgG autoantibody levels in serum samples obtained from TBI patients (circle labeled) at day 30 and day 90 post injury (N=142) are lower in comparison with healthy controls (cross labeled) (N=21). Figure 4B shows the separation performance by receivers operating characteristic (ROC) curves of anti-CMV IgG autoantibody levels.

EXAMPLE 4: The prediction of the clinical status of TBI patients at day 90 post injury, based on the anti-TNFRSF12A (SEQ ID No: 104) IgM autoantibody levels in serum samples obtained from TBI patients at day 30 post injury.

As shown in Figure 5A, the levels of anti-TNFRSF12A (SEQ ID No:104) IgM autoantibody in serum samples obtained from TBI patients at day 30 post injury can be used for the prediction of the clinical status (GOSE <8 or GOSE =8) of TBI patients at day 90 post injury. Figure 5B shows the separation performance by receivers operating characteristic (ROC) curves of anti-TNFRSF12A IgM autoantibody levels.

EXAMPLE 5: Combination measurement of the levels of autoantibodies and biomarkers in serum samples obtained from TBI patients as compared to healthy controls

To determine whether combination measurement of the levels of antibodies and biomarkers in serum samples can differentiate between TBI patients and healthy controls, a combined analysis was conducted. Serum samples obtained from TBI patients at time 0 (t0, N=85) were compared with serum samples obtained from healthy control (HC, N=21). The analysis was based on 464 iChip features (232 antigens, IgM and IgG) and four ELISA features. iChip data is based on average of two block replicates, following correction procedure. ELISA features were selected based on data availability; only features with data available for > 80% of the iChip samples were used. Samples with missing ELISA data were removed from the analysis.

Figure 6 shows the area under the Receiver Operating Characteristics (ROC) curves of six classification methods (SVM, LR, QDA, CART, RF and LDA) based on 100 iterations of 70:30 cross validation. Features were ranked according to their median scoring or frequency of model inclusion, depending on the method.

Using the LDA classification method revealed that the top six features above the random background level are the biomarkers: GFAP and SNCB in combination with the autoantibodies: anti-MBP in vitro citrullinated (SEQ ID No: 2) IgM, anti-GFAP (SEQ ID No: 14) IgM, anti-ICAM5 (SEQ ID No: 28) IgM, and anti- BDNF (SEQ ID No: 42) IgM.

Using the QDA classification method revealed that the top three features above the random background level are the biomarkers: GFAP and SNCB in combination with the autoantibodies: anti-MBP in vitro citrullinated (SEQ ID No: 2).

EXAMPLE 6: Combination measurement of the levels of antibodies and biomarkers in serum samples obtained from TBI patients with intracranial hemorrhage on head CT as compared to TBI patients with normal CT

To determine whether combination measurement of the levels of antibodies and biomarkers in serum samples can differentiate between TBI patients with intracranial hemorrhage on head CT and those with normal CT, a combined analysis was conducted. Serum samples obtained from TBI patients at time 0 (t0) with abnormal CT were compared

with samples obtained from TBI patients at time 0 (t0) with normal CT. Analysis was based on 464 iChip features (232 antigen, IgM and IgG) and four ELISA features. iChip data is based on average of two block replicates, following correction procedure. ELISA features were selected based on data availability; only features with data available for > 80% of the iChip samples were used. Samples with missing ELISA data were removed from the analysis.

Figure 7 shows ROC curves of six classification methods (SVM, LR, QDA, CART, RF and LDA), based on 100 iterations of 70:30 cross validation. Features were ranked according to their median scoring or frequency of model inclusion, depending on the method.

Using the LDA classification method revealed that the top five features above the random background level are the biomarker: SNCB in combination with the autoantibodies: anti-Collagen IV (SEQ ID No: 66) IgG, anti-Oligo24 (SEQ ID No: 44) IgM, anti-EBV IgM and anti-Collagen II (SEQ ID No: 102) IgG.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without undue experimentation and without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation. The means, materials, and steps for carrying out various disclosed functions may take a variety of alternative forms without departing from the invention.

CLAIMS

1. A method for diagnosing brain injury in a subject, the method comprising the steps of:

(i) obtaining a sample from the subject;

(ii) determining the reactivity of antibodies in the sample to at least one antigen selected from the group consisting of SEQ ID NOS: 1-115, isoforms thereof, post-translationally modified forms thereof, fragments thereof, or any combinations thereof; and

(iii) comparing the reactivity of antibodies in the sample to a reactivity of a healthy control;

wherein a significantly different reactivity of the antibodies in the sample compared to the reactivity of the healthy control is an indication that the subject is having brain injury.

2. The method of claim 1, wherein the reactivity of antibodies comprises IgG and IgM reactivities.

3. The method of claim 1, wherein the significantly different reactivity of the antibodies in the sample comprises differential IgG and IgM reactivities.

4. The method of claim 1, wherein said brain injury is selected from the group consisting of: concussions, chronic traumatic encephalopathy, mild traumatic brain injuries, moderate traumatic brain injuries, severe traumatic brain injuries, head trauma, concussive blasts and brain neurodegenerative condition.

5. The method of claim 4, wherein the brain neurodegenerative condition further comprises loss of memory or motor function.

6. The method of claim 5, wherein said neurodegenerative condition is selected from the group consisting of: Alzheimer's Disease, Huntington's Disease, Parkinson's Disease, demyelinating disease, HTLV-1-associated myelopathy (HAM), multiple sclerosis (MS), amyotrophic lateral sclerosis, pathological neurological symptoms after injury or trauma, encephalopathy and viral encephalopathy.

7. The method of claim 1, wherein the sample is selected from the group consisting of blood, serum, plasma, cerebrospinal fluid (CSF), urine and saliva sample.

8. The method of claim 1, wherein the sample is a serum sample.

9. The method of claim 1, wherein the reactivity of a healthy control is selected from the group consisting of a reactivity of at least one healthy individual, a baseline sample from the same individual, a panel of control samples from a set of healthy individuals, and a stored set of data from healthy control individuals.

10. The method of claim 1, comprising determining the reactivity of antibodies in the sample to a plurality of the antigens.

11. The method of claim 10, wherein the plurality of antigens is used in the form of an antigen probe set, an antigen array, or an antigen chip.

12. The method of claim 1, further comprising measuring the levels of one or more biomarkers in the sample; and comparing the levels of the one or more biomarkers with predefined levels of the same biomarkers that correlate to a subject having brain injury and predefined levels of the same biomarkers that correlate to a healthy control, wherein a correlation to one of the predefined levels provides the diagnosis.

13. The method of claim 12, wherein the one or more biomarkers is selected from the group consisting of glial fibrillary acidic protein (GFAP), Synuclein beta (Sncb), Metallothionein-3 (MT3), Neurogranin (NRGN), intercellular adhesion molecule-5 (ICAM5) and Brain derived neurotrophic factor (BDNF), or citrullinated forms thereof.

14. An antigen probe set comprising a plurality of antigen probes selected from the group consisting of SEQ ID NOs: 1-115, isoforms thereof, post-translationally modified forms thereof, fragments thereof, or any combinations thereof.

15. An article of manufacture comprising the antigen probe set of claim 14.

16. The article of manufacture of claim 15, further comprising one or more biomarkers selected from the group consisting of glial fibrillary acidic protein (GFAP), Synuclein beta (Sncb), Metallothionein-3 (MT3), Neurogranin (NRGN), intercellular adhesion molecule-5 (ICAM5) and Brain derived neurotrophic factor (BDNF), or citrullinated forms thereof.

17. The article of manufacture of claim 15, in the form of an antigen probe array or in the form of an antigen chip or in the form of a dipstick or in the form of a lateral flow test.

18. The article of manufacture of claim 17, in the form of a kit further comprising means for performing the method of claim 1, or instructions for use of the kit for diagnosing brain injury.

19. A method for qualifying brain injury status in a subject the method comprising the steps of:

(i) obtaining a sample from the subject;

(ii) determining the reactivity of antibodies in the sample to at least one antigen selected from the group consisting of SEQ ID NOS: 1-115, isoforms thereof, post-translationally modified forms thereof, fragments thereof, or any combinations thereof; and

comparing the reactivity of antibodies in the sample to a predefined reactivity that correlate to one or more brain injury statuses selected from the group consisting of having brain injury, not having brain injury, predisposition to brain injury, sub-acute brain injury, acute brain injury, post-acute brain injury, progressing brain injury, regressing brain injury, subclinical brain injury, mild brain injury, moderate brain injury, severe brain injury and chronic brain injury, wherein a correlation to one of the predefined reactivities determines the brain injury status of the subject.

20. The method of claim 19, further comprising measuring the levels of one or more biomarkers in the sample; and comparing the levels of the one or more biomarkers with predefined levels of the same biomarkers that correlate to one or more brain injury statuses, wherein a correlation to one of the predefined levels determines the brain injury status of the subject.

21. The method of claim 20, wherein the one or more biomarkers is selected from the group consisting of glial fibrillary acidic protein (GFAP), Synuclein beta (Sncb), Metallothionein-3 (MT3), Neurogranin (NRGN), intercellular adhesion molecule-5 (ICAM5) and Brain derived neurotrophic factor (BDNF), or citrullinated forms thereof.

22. A method of detecting recovery from brain injury in a subject, the method comprising the steps of:

(i) obtaining a sample from the subject;

(ii) determining the reactivity of antibodies in the sample to at least one antigen selected from the group consisting of SEQ ID NOS: 10, 61, 104, isoforms thereof, post-translationally modified forms thereof, fragments thereof, or any combinations thereof; and

(iii) comparing the reactivity of antibodies in the sample to a predefined reactivity threshold; wherein a significantly different reactivity of the antibodies in the sample compared to the predefined reactivity threshold is indicative of recovery from brain injury in said subject.

23. The method of claim 22, wherein the reactivity of antibodies comprises IgG and IgM reactivities.

24. The method of claim 22, wherein the significantly different reactivity of the antibodies in the sample comprises differential IgG and IgM reactivities.

25. The method of claim 22, further comprising measuring the levels of one or more biomarkers in the sample; and comparing the levels of the one or more biomarkers with predefined levels of the same biomarkers that correlates with recovery from brain injury, wherein a correlation to one of the predefined levels is indicative of recovery from brain injury of the subject.

26. The method of claim 25, wherein the one or more biomarkers is selected from the group consisting of glial fibrillary acidic protein (GFAP), Synuclein beta (Sncb), Metallothionein-3 (MT3), Neurogranin (NRGN), intercellular adhesion molecule-5 (ICAM5) and Brain derived neurotrophic factor (BDNF), or citrullinated forms thereof.

27. The method of any one of claims 1, 19 or 22, wherein said comparison is conducted by using at least one classifier algorithm.

28. The method of claim 27, wherein said at least one classifier algorithm is selected from the group consisting of a decision tree classifier, logistic regression classifier (LR), nearest neighbor classifier, neural network classifier, Gaussian mixture model (GMM), Support Vector Machine (SVM) classifier, nearest centroid classifier, linear regression classifier, linear discriminant analysis (LDA) classifier, quadratic discriminant analysis (QDA) classifier and random forest classifier.

Fig. 1

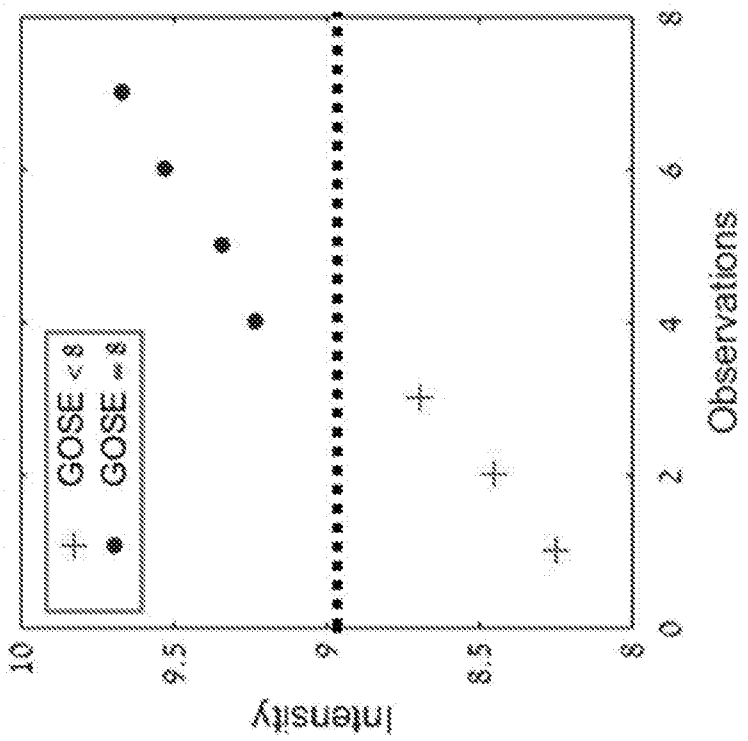


Fig. 2

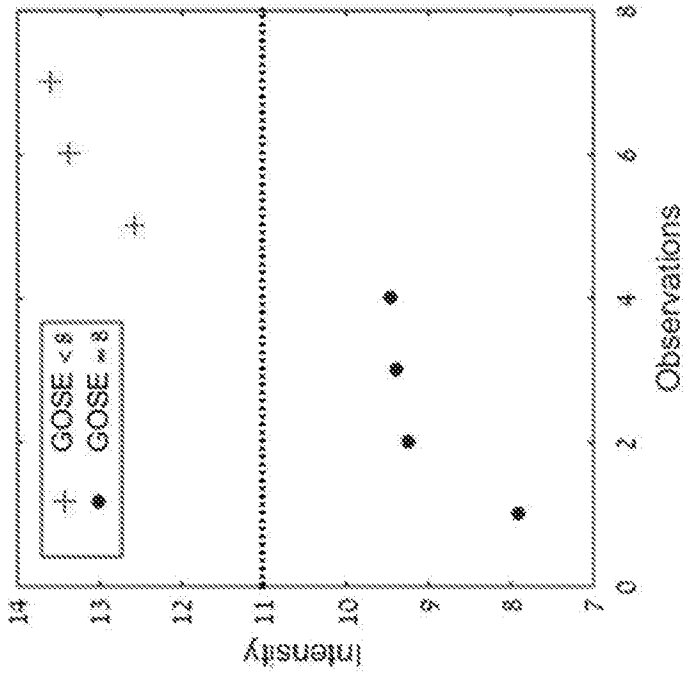


Fig. 3

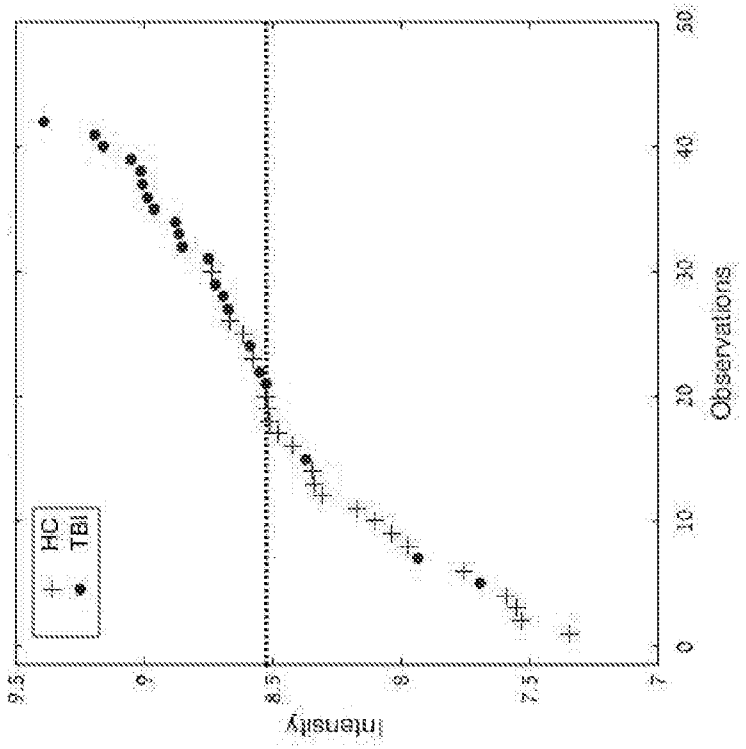


Fig. 4B

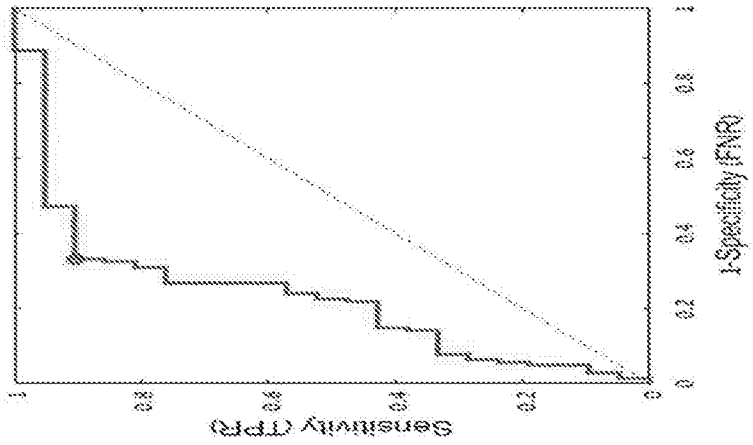


Fig. 4A

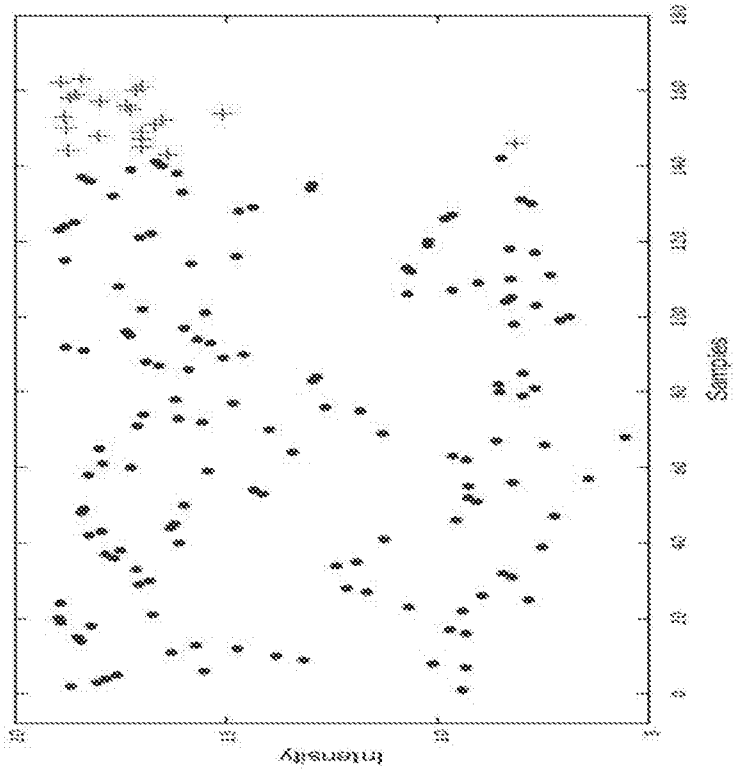


Fig. 5B

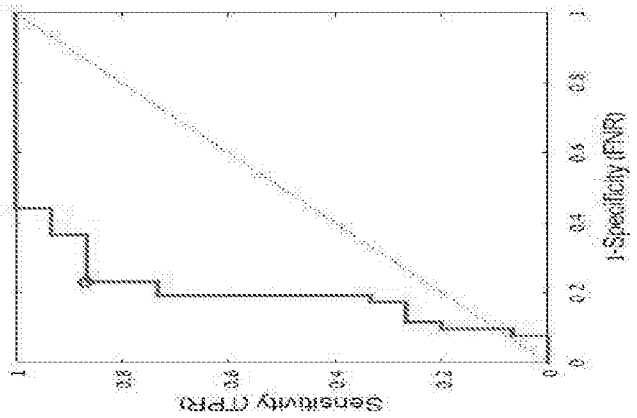


Fig. 5A

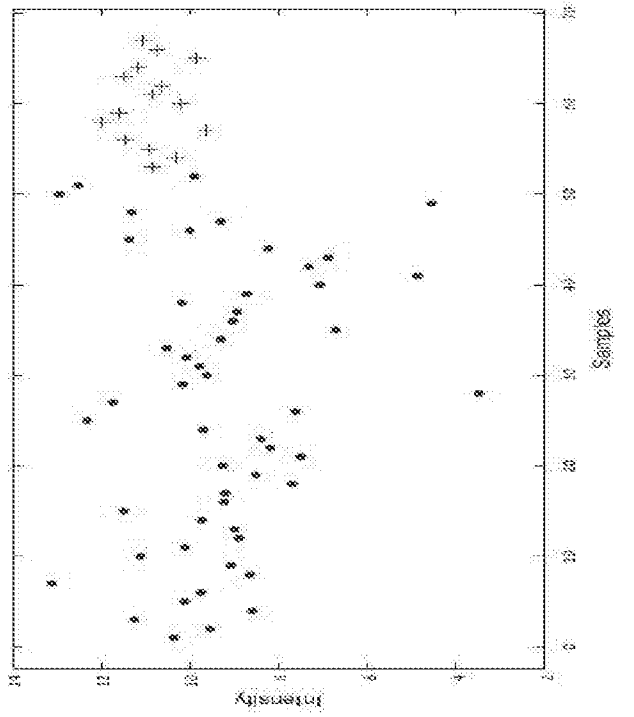
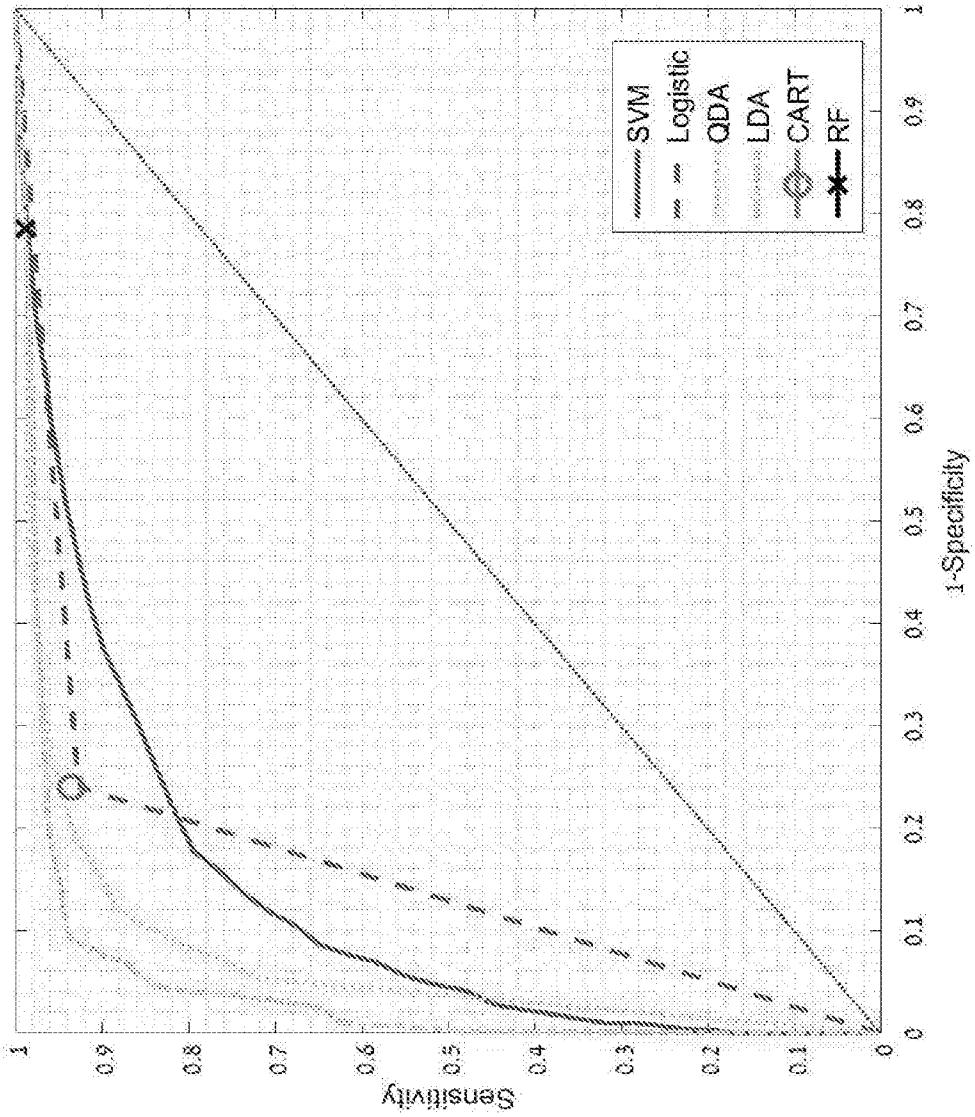


Fig. 6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LDA
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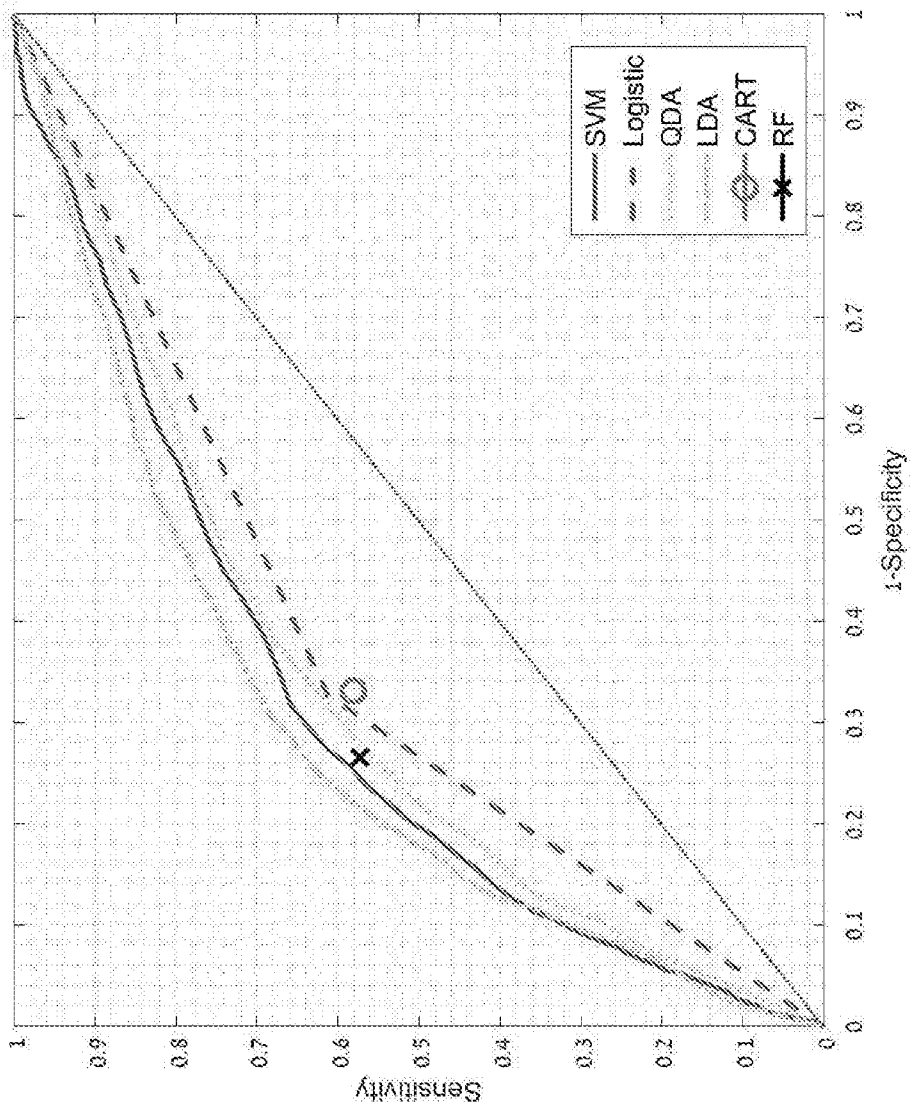
Fig. 7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LDA
CART
RF
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL2016/050108

A. CLASSIFICATION OF SUBJECT MATTER
IPC (2016.01) G01N 33/50, G01N 33/53, C07K 14/46

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)
IPC (2016.01) G01N, C07K

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)
Databases consulted: THOMSON INNOVATION, Esp@cenet, Google Patents, CAPLUS, BIOSIS, EMBASE, MEDLINE, WPI Data, PubMed, Google Scholar
Search terms used: "traumatic brain injury"; autoantibodies; "brain injury"; "myelin basic protein"; mbp;

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Consequences of Repeated Blood-Brain Barrier Disruption in Football Players. PLoS ONE 8 (3): e56805. doi:10.1371/journal.pone.0056805 Nicola Marchi, Jeffrey J. Bazarian, Vikram Puvenna, Mattia Janigro, Chaitali Ghosh, Jianhui Zhong, Tong Zhu, Eric Blackman, Desiree Stewart, Jasmina Ellis, Robert Butler, Damir Janigro 06 Mar 2013 (2013/03/06) The whole document, especially the abstract, Figure 2 and Figure 3	1-21
X	Human Traumatic Brain Injury Induces Autoantibody Response against Glial Fibrillary Acidic Protein and Its Breakdown Products. PLoS ONE 9(3): e92698. doi:10.1371/journal.pone.0092698 Zhang Z, Zoltewicz JS, Mondello S, Newsom KJ, Yang Z, et al. 24 Mar 2014 (2014/03/24) The whole document, especially the abstract, figure 2.	1-21
X	Roles of autoantibodies in central nervous system injury. Discovery medicine, 11(60), 395-402. http://www.discoverymedicine.com/Yi-Zhang/2011/05/10/roles-of-autoantibodies-in-central-nervous-system-injury/ Roles of autoantibodies in central nervous system injury. Discovery medicine, 11(60), 395-402. http://www.discoverymedicine.com/Yi-Zhang/2011/05/10/roles-of-autoantibodies-in-central-nervous-system-injury/ 10 May 2011 (2011/05/10) Table 1	1-21

[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
13 Jun 2016	15 Jun 2016
Name and mailing address of the ISA: Israel Patent Office Technology Park, Bldg.5, Malcha, Jerusalem, 9695101, Israel Facsimile No. 972-2-5651616	Authorized officer PACE Umberto Telephone No. 972-2-5651625

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL2016/050108

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	[Immunological markers of severity and outcome of traumatic brain injury]. Zhurnal nevrologii i psikhiatrii imeni SS Korsakova/Ministerstvo zdravookhraneniia i meditsinskoi promyshlennosti Rossiiskoi Federatsii, Vserossiiskoe obshchestvo nevrologov [i] Vserossiiskoe obshchestvo psikhiatrov, 111(7), 61-65. Ngankam, L., Kazantseva, N. V., & Gerasimova, M. M. 31 Dec 2010 (2010/12/31) Abstract	1-21
X	WO 03042701 A2 The Hospital for sick children 22 Mar 2003 (2003/03/22) abstract, claims 1 and	1-21
X	US 2015031048 A1 The Johns Hopkins University 29 Jan 2015 (2015/01/29) Paragraph 38, claims 51-54	1-21
X	US 2013022982 A1 Wang et al. 24 Jan 2013 (2013/01/24) Abstract, paragraph 6, claims.	1-21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL2016/050108

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:

See extra sheet.

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 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.:
1-21

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.

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:

Invention/s 1	A method of diagnosing or qualifying brain injury comprising the step of determining the reactivity of antibodies to the antigen having SEQ ID NO: 1 in a sample from an injured individual. An antigen probe set comprising the antigen having SEQ ID NO: 1 and an article of manufacture comprising said probe set.	Claim/s 1-21
Invention/s 2 - 115	A method of diagnosing or qualifying brain injury comprising the step of determining the reactivity of antibodies to one antigen having SEQ ID NOS: 2-115 in a sample from an injured individual. An antigen probe set comprising one antigen having SEQ ID NOS: 2-115 and an article of manufacture comprising said probe set.	Claim/s 1-12
Invention/s 116 - 118	A method of detecting recovery from brain injury comprising the step of determining the reactivity of antibodies to one antigen having SEQ ID NOS: 10, 61 or 104 in a sample from an injured individual.	Claim/s 22-28

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IL2016/050108

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专利名称(译)	用于诊断脑损伤或神经变性的方法和组合物		
公开(公告)号	EP3254106A4	公开(公告)日	2018-10-03
申请号	EP2016746229	申请日	2016-02-01
[标]发明人	SOREK RACHEL JAKOBI KEREN EDMONDS DONNA		
发明人	SOREK, RACHEL JAKOBI, KEREN EDMONDS, DONNA		
IPC分类号	G01N33/50 G01N33/53 C07K14/46		
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优先权	62/112189 2015-02-05 US		
其他公开文献	EP3254106A1		
外部链接	Espacenet		

摘要(译)

用于诊断脑损伤，神经变性的方法和组合物;或者提供在受试者中的倾向。特别地，本发明涉及在受试者中用于诊断脑损伤，神经变性或其易感性的特异性抗原抗体反应性。