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(54) Title: METHODS AND COMPOSITIONS FOR DIAGNOSIS AND PROGNOSIS OF STROKE OR OTHER CEREBRAL INJURY

(57) Abstract: The present invention relates to methods and compositions for monitoring, diagnosis, prognosis, and determination of treatment regimens in stroke patients and in patients at risk for stroke. In particular, the invention relates to using assays that detect one or more biomarkers as diagnostic and prognostic biomarker assays in such patients.

METHODS AND COMPOSITIONS FOR DIAGNOSIS AND PROGNOSIS OF STROKE OR OTHER CEREBRAL INJURY

[0001] The present application claims priority to U.S. Provisional Patent Applications 61/637,813 filed April 24, 2012; 61/637,815 filed April 24, 2012; 61/674,650 filed July 23, 2012; 61/674,656 filed July 23, 2012; and 61/674,660 filed July 23, 2012, each of which is hereby incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

[0002] The following discussion of the background of the invention is merely provided to aid the reader in understanding the invention and is not admitted to describe or constitute prior art to the present invention.

[0003] Stroke is a manifestation of vascular injury to the brain which is commonly secondary to atherosclerosis or hypertension, and is the third leading cause of death (and the second most common cause of neurologic disability) in the United States. Stroke can be categorized into two broad types, "ischemic stroke" and "hemorrhagic stroke." Additionally, a patient may experience transient ischemic attacks, which share the same underlying cause as strokes and which cause the same symptoms. The similarity of these acute clinical syndromes made it difficult to differentiate them; they were distinguished on the basis of an arbitrary criterion for the duration of symptoms. While the damage from a TIA may be just as severe as a stroke, the symptoms of a TIA can resolve within a few minutes or 24 hours. For purposes of the present invention, ischemic stroke and hemorrhagic stroke and TIA will all be referred to as "strokes."

[0004] Ischemic brain injury encompasses thrombotic, embolic, lacunar and hypoperfusion types of strokes. Thrombi are occlusions of arteries created *in situ* within the brain, while emboli are occlusions caused by material from a distant source, such as the heart and major vessels, often dislodged due to myocardial infarct or atrial fibrillation. Less frequently, thrombi may also result from vascular inflammation due to disorders such as meningitis. Thrombi or emboli can result from atherosclerosis or other disorders, for example, arteritis, and lead to physical obstruction of arterial blood supply to the brain. Lacunar stroke refers to an infarct within non-cortical regions of the brain. Hypoperfusion embodies diffuise injury caused by non-localized cerebral ischemia, typically caused by myocardial infarction and arrhythmia.

[0005] The onset of ischemic brain injury is often abrupt, and can become an "evolving stroke" manifested by neurologic deficits that worsen over a 24-48 hour period. In evolving stroke, "stroke-associated symptom(s)" commonly include unilateral neurologic dysfunction that extends progressively, without producing headache or fever. Evolving stroke may also become a "completed stroke," in which symptoms develop rapidly and are maximal within a few minutes.

[0006] Hemorrhagic stroke is caused by intracerebral or subarachnoid hemorrhage, i.e., bleeding into brain tissue, following blood vessel rupture within the brain. Intracerebral and subarachnoid hemorrhages are subsets of a broader category of hemorrhage referred to as intracranial hemorrhage. Intracerebral hemorrhage is typically due to chronic hypertension, and a resulting rupture of an arteriosclerotic vessel. Stroke-associated symptom(s) of intracerebral hemorrhage are abrupt, with the onset of headache and steadily increasing neurological deficits. Nausea, vomiting, delirium, seizures and loss of consciousness are additional common stroke-associated symptoms.

[0007] In contrast, most subarachnoid hemorrhage is caused by head trauma or aneurysm rupture which is accompanied by high pressure blood release which also causes direct cellular trauma. Prior to rupture, aneurysms may be asymptomatic, or occasionally associated with tension or migraine headaches. However, headache typically becomes acute and severe upon rupture, and may be accompanied by varying degrees of neurological deficit, vomiting, dizziness, and altered pulse and respiratory rates.

[0008] Concepts of brain ischemia and the temporal correlation with clinical events have changed considerably on the basis of studies using computed tomography (CT), magnetic resonance imaging (MRI), positron-emission tomography, and other imaging techniques. At most institutions, CT of the brain is performed as part of the initial evaluation of a patient with suspected stroke. The main advantage of this imaging modality is its widespread availability and sensitivity for hemorrhage. However, it is insensitive to early ischemic changes during acute cerebral ischemia. Several technologies based on MRI have shown promise for the early diagnosis of stroke. However, as a practical issue, most hospitals lack the necessary specialized MRI services in the acute setting.

[0009] Another approach to the diagnosis of acute stroke has been the evaluation of biomarkers in body fluid samples such as blood. By way of example, acute stroke has

been associated with serum elevations of numerous biomarkers related to inflammation, coagulation, and glial cell damage in a variety of research studies. To date, however, no biomarker test has been demonstrated to possess the requisite sensitivity and specificity to allow it to function as a useful clinical diagnostic.

[0010] There remains a need in the art for a rapid, objective, clinically accurate, available diagnostic tool for aiding in the diagnosis and care of stroke patients.

BRIEF SUMMARY OF THE INVENTION

[0011] The present invention relates to the identification and use of diagnostic markers for stroke and cerebral injury. The methods and compositions described herein can meet the need in the art for rapid, sensitive and specific diagnostic assay to be used in the diagnosis and differentiation of various forms of stroke. Moreover, the methods and compositions of the present invention can also be used to facilitate the treatment of stroke patients and the development of additional diagnostic and/or prognostic indicators.

[0012] In various aspects, the invention relates to materials and procedures for identifying markers that are associated with the diagnosis, prognosis, or differentiation of stroke and/or TIA in a patient; to using such markers in diagnosing and treating a patient and/or to monitor the course of a treatment regimen; to using such markers to identify subjects at risk for one or more adverse outcomes related to stroke and/or TIA; and for screening compounds and pharmaceutical compositions that might provide a benefit in treating or preventing such conditions.

[0013] In a first aspect, the invention discloses methods for determining a diagnosis or prognosis related to cerebral injury, or for differentiating between types of strokes and/or TIA. As described herein, measurement of one or more biomarkers selected from the group consisting of Agouti-related protein, Alpha-2 macroglobulin, Alpha-fetoprotein, Amphiregulin, Angiopoietin-1 receptor, Angiopoietin-related protein 3, Angiopoietin-related protein 4, Angiopoietin-related protein 6, Bone morphogenetic protein 7, Cadherin-3, Cancer Antigen 15-3, Cancer Antigen 19-9, Carcinoembryonic antigen-related cell adhesion molecule 5, C-C motif chemokine 1, C-C motif chemokine 13, C-C motif chemokine 15, C-C motif chemokine 17, C-C motif chemokine 19, C-C motif chemokine 26, C-C motif chemokine 8, Choriogonadotropin subunit beta, Clusterin, C-X-C motif chemokine 11, C-X-C motif chemokine 6, C-X-C motif chemokine 9, Cyclin-dependent kinase inhibitor 1, Endoglin, Epiregulin, Epithelial cell

adhesion molecule, Erythropoietin, Fatty acid-binding protein, liver, Fibroblast growth factor 19, Fibroblast growth factor 21, Fibroblast growth factor 23, Follistatin, Follitropin subunit beta, Galectin-3, Glial cell line-derived neurotrophic factor, Heat shock protein beta-1, Heparin-binding EGF-like growth factor, Heparin-binding growth factor 1, Hepatitis A virus cellular receptor 1, Hepatocyte growth factor receptor, Insulin receptor substrate 1, Insulin-like growth factor-binding protein 1, Insulin-like growth factor-binding protein 2, Insulin-like growth factor-binding protein 4, Intercellular adhesion molecule 3, Interleukin-1 receptor type I, Interleukin-1 receptor type II, Interleukin-11, Interleukin-21, Interleukin-28A, Interleukin-29, Interleukin-33, Interleukin-4 receptor alpha chain, Interleukin-6 receptor subunit alpha, Interleukin-6 receptor subunit beta, Keratin, type I cytoskeletal 19 (aa311-367), Kit ligand, Lutropin subunit beta, Lymphotactin, Macrophage colony-stimulating factor 1, Matrilysin, Metalloproteinase inhibitor 4, Growth-regulated alpha, beta, and gamma proteins (total), Mucin-16, Neprilysin, Neuronal cell adhesion molecule, NF-kappa-B inhibitor alpha, Osteocalcin, Oxidized low-density lipoprotein receptor 1, Parathyroid hormone, Platelet endothelial cell adhesion molecule, Probetacellulin, Pro-interleukin-16, Prolactin, Proprotein convertase subtilisin/kexin type 9, Prostate-specific antigen, Protein NOV homolog, Protransforming growth factor alpha, Serum amyloid P-component, Somatotropin, Stromal cell-derived factor 1, Thymic stromal lymphopoietin, Thyrotropin subunit beta, Transmembrane glycoprotein NMB, Tumor necrosis factor receptor superfamily member 8, Vascular endothelial growth factor D, Vascular endothelial growth factor receptor 1, Vascular endothelial growth factor receptor 2, Vascular endothelial growth factor receptor 3, and WAP four-disulfide core domain protein 2 (each referred to herein for convenience as a "stroke biomarker") can be used for diagnosis, prognosis, risk stratification, staging, monitoring, categorizing and determination of further diagnosis and treatment regimens in patients having or suspected of having a cerebral injury, including ischemic stroke, hemorrhagic stroke, TIA, or traumatic brain injury.

[0014] In a related aspect, the invention discloses methods for determining a diagnosis or prognosis related to cerebral injury, or for differentiating between types of strokes and/or TIA. As described herein, measurement of one or more of, preferably a plurality of, and most preferably each of, biomarkers selected from the group consisting of eotaxin, epidermal growth factor receptor, S100A12, TIMP-4, and prolactin (each referred to herein for convenience as a "stroke biomarker") can be used for diagnosis,

prognosis, risk stratification, staging, monitoring, categorizing and determination of further diagnosis and treatment regimens in patients having or suspected of having a cerebral injury, including ischemic stroke, hemorrhagic stroke, TIA, or traumatic brain injury.

[0015] The stroke biomarkers of the present invention may be used, individually or in panels comprising a plurality of stroke biomarkers. The presence or amount of such marker(s) in a sample obtained from the subject can be used to rule in or rule out one or more of the following: traumatic brain injury, stroke, thrombotic stroke, embolic stroke, lacunar stroke, hypoperfusion, intracerebral hemorrhage, and subarachnoid hemorrhage, thereby either providing a diagnosis (rule-in) and/or excluding a diagnosis (rule-out).

[0016] As noted above, the present invention relates to methods for evaluating a stroke patient or a patient being evaluated for a possible diagnosis. These methods comprise performing an assay method that is configured to detect one or more biomarkers selected from the group consisting of Agouti-related protein, Alpha-2 macroglobulin, Alpha-fetoprotein, Amphiregulin, Angiopoietin-1 receptor, Angiopoietin-related protein 3, Angiopoietin-related protein 4, Angiopoietin-related protein 6, Bone morphogenetic protein 7, Cadherin-3, Cancer Antigen 15-3, Cancer Antigen 19-9, Carcinoembryonic antigen-related cell adhesion molecule 5, C-C motif chemokine 1, C-C motif chemokine 13, C-C motif chemokine 15, C-C motif chemokine 17, C-C motif chemokine 19, C-C motif chemokine 26, C-C motif chemokine 8, Choriogonadotropin subunit beta, Clusterin, C-X-C motif chemokine 11, C-X-C motif chemokine 6, C-X-C motif chemokine 9, Cyclin-dependent kinase inhibitor 1, Endoglin, Epiregulin, Epithelial cell adhesion molecule, Erythropoietin, Fatty acid-binding protein, liver, Fibroblast growth factor 19, Fibroblast growth factor 21, Fibroblast growth factor 23, Follistatin, Follitropin subunit beta, Galectin-3, Glial cell line-derived neurotrophic factor, Heat shock protein beta-1, Heparin-binding EGF-like growth factor, Heparin-binding growth factor 1, Hepatitis A virus cellular receptor 1, Hepatocyte growth factor receptor, Insulin receptor substrate 1, Insulin-like growth factor-binding protein 1, Insulin-like growth factor-binding protein 2, Insulin-like growth factor-binding protein 4, Intercellular adhesion molecule 3, Interleukin-1 receptor type I, Interleukin-1 receptor type II, Interleukin-11, Interleukin-21, Interleukin-28A, Interleukin-29, Interleukin-33, Interleukin-4 receptor alpha chain, Interleukin-6 receptor subunit alpha, Interleukin-6 receptor subunit beta, Keratin, type I cytoskeletal 19 (aa311-367), Kit ligand, Lutropin subunit beta,

Lymphotactin, Macrophage colony-stimulating factor 1, Matrilysin, Metalloproteinase inhibitor 4, Growth-regulated alpha, beta, and gamma proteins (total), Mucin-16, Neprilysin, Neuronal cell adhesion molecule, NF-kappa-B inhibitor alpha, Osteocalcin, Oxidized low-density lipoprotein receptor 1, Parathyroid hormone, Platelet endothelial cell adhesion molecule, Probetacellulin, Pro-interleukin-16, Prolactin, Proprotein convertase subtilisin/kexin type 9, Prostate-specific antigen, Protein NOV homolog, Protransforming growth factor alpha, Serum amyloid P-component, Somatotropin, Stromal cell-derived factor 1, Thymic stromal lymphopoietin, Thyrotropin subunit beta, Transmembrane glycoprotein NMB, Tumor necrosis factor receptor superfamily member 8, Vascular endothelial growth factor D, Vascular endothelial growth factor receptor 1, Vascular endothelial growth factor receptor 2, Vascular endothelial growth factor receptor 3, and WAP four-disulfide core domain protein 2, the results of which assay(s) is/are then correlated to the status of the patient.

[0017] The present invention also relates to methods for evaluating a stroke patient or a patient being evaluated for a possible diagnosis. These methods comprise performing an assay method that is configured to detect one or more of, preferably a plurality of, and most preferably each of, biomarkers selected from the group consisting of eotaxin, epidermal growth factor receptor, S100A12, TIMP-4, and prolactin, the results of which assay(s) is/are then correlated to the status of the patient.

[0018] This correlation to status may include one or more of the following: diagnosis of stroke; diagnosis of stroke and indication if an acute stroke has occurred; diagnosis of stroke and indication if an non-acute stroke has occurred; diagnosis of stroke, indication if an acute stroke has occurred, and indication if an non-acute stroke has occurred; diagnosis of stroke and indication if an ischemic stroke has occurred; diagnosis of stroke and indication if a hemorrhagic stroke has occurred; diagnosis of stroke, indication if an ischemic stroke has occurred and indication if a hemorrhagic stroke has occurred; diagnosis of stroke and prognosis of a subsequent adverse outcome; diagnosis of stroke and prognosis of a subsequent cerebral vasospasm; diagnosis of stroke, indication if a hemorrhagic stroke has occurred, prognosis of a subsequent cerebral vasospasm; indication if a traumatic brain injury has occurred; indication of a prognosis resulting from a cerebral injury selected from the group consisting of ischemic stroke, hemorrhagic stroke, TIA, and traumatic brain injury.

[0019] In certain embodiments, the methods for evaluating a patient described herein are methods for risk stratification of the patient; that is, assigning a likelihood of one or more future changes in health status to the patient. In these embodiments, the assay result(s) is/are correlated to one or more such future changes. A level or a change in level of one or more stroke biomarkers, which in turn is(are) associated with an increased probability of morbidity or death, is referred to as being "associated with an increased predisposition to an adverse outcome" in a patient. Preferred prognostic markers can predict the likelihood of the onset of delayed neurologic deficits in a patient after stroke or other cerebral injury, predict the likelihood of a subsequent stroke, or predict the likelihood of death.

[0020] In such risk stratification embodiments, preferably the likelihood or risk assigned is that an event of interest is more or less likely to occur within 180 days of the time at which the body fluid sample is obtained from the patient. In particularly preferred embodiments, the likelihood or risk assigned relates to an event of interest occurring within a shorter time period such as 18 months, 120 days, 90 days, 60 days, 45 days, 30 days, 21 days, 14 days, 7 days, 5 days, 96 hours, 72 hours, 48 hours, 36 hours, 24 hours, 12 hours, or less. A risk at 0 hours of the time at which the body fluid sample is obtained from the stroke patient is equivalent to diagnosis of a current condition.

[0021] For a positive going marker, an increased likelihood of the occurrence of a diagnosis is assigned to the patient when the measured concentration is above the threshold (relative to the likelihood assigned when the measured concentration is below the threshold); alternatively, when the measured concentration is below the threshold, an increased likelihood of the nonoccurrence of a diagnosis may be assigned to the patient (relative to the likelihood assigned when the measured concentration is above the threshold). For a negative going marker, an increased likelihood of the occurrence of a diagnosis is assigned to the patient when the measured concentration is below the threshold (relative to the likelihood assigned when the measured concentration is above the threshold); alternatively, when the measured concentration is above the threshold, an increased likelihood of the nonoccurrence of a diagnosis may be assigned to the patient (relative to the likelihood assigned when the measured concentration is below the threshold).

[0022] In certain embodiments, a biomarker or panel of biomarkers is correlated to a condition or disease by merely its presence or absence. In other embodiments, a

threshold level of a diagnostic or prognostic indicator can be established, and the level of the indicator in a patient sample can simply be compared to the threshold level. A variety of methods may be used by the skilled artisan to arrive at a desired threshold value for use in these methods. For example, for a positive going marker the threshold value may be determined from a population of patients not having had a stroke by selecting a concentration representing the 75^{th}, 85^{th}, 90^{th}, 95^{th}, or 99^{th} percentile of a stroke biomarker or biomarkers measured in such "normal" patients. Alternatively, the threshold value may be determined from a "diseased" population of patients by selecting a concentration representing the 75^{th}, 85^{th}, 90^{th}, 95^{th}, or 99^{th} percentile of a biomarker or biomarkers measured in patients suffering from a stroke or other cerebral injury.

[0023] Alternatively, the threshold value may be determined from a "diseased" population of stroke patients having a predisposition for an outcome such as death, worsening disease, *etc.*), by selecting a concentration representing the 75^{th}, 85^{th}, 90^{th}, 95^{th}, or 99^{th} percentile of a biomarker or biomarkers measured in patients suffering from a stroke or other cerebral injury and who later suffered from the outcome of interest.

[0024] In another alternative, the threshold value may be determined from a prior measurement of a biomarker or biomarkers in the same patient; that is, a temporal change in the level of a biomarker or biomarkers in the same patient may be used to assign a diagnosis or a prognosis to the patient. For example, a diagnostic indicator may be determined at an initial time, and again at a second time. In such embodiments, an increase in the marker from the initial time to the second time may be diagnostic of a particular type of stroke, or a given prognosis.

[0025] The foregoing discussion is not meant to imply, however, that the stroke biomarkers of the present invention must be compared to corresponding individual thresholds. Methods for combining assay results can comprise the use of multivariate logistical regression, loglinear modeling, neural network analysis, n-of-m analysis, decision tree analysis, calculating ratios of markers, *etc.* This list is not meant to be limiting. In these methods, a composite result which is determined by combining individual markers may be treated as if it is itself a marker; that is, a threshold may be determined for the composite result as described herein for individual markers, and the composite result for an individual patient compared to this threshold.

[0026] The ability of a particular test to distinguish two populations can be established using ROC analysis. For example, ROC curves established from a "first" subpopulation which has a particular disease (or which is predisposed to some outcome), and a "second" subpopulation which does not have the disease (or is not so predisposed) can be used to calculate a ROC curve, and the area under the curve provides a measure of the quality of the test. Preferably, the tests described herein provide a ROC curve area greater than 0.5, preferably at least 0.6, more preferably 0.7, still more preferably at least 0.8, even more preferably at least 0.9, and most preferably at least 0.95.

[0027] In certain aspects, the measured concentration of one or more stroke biomarkers, or a composite of such markers, may be treated as continuous variables. For example, any particular concentration can be converted into a corresponding probability of existing disease, of a future outcome for the stroke patient, or mortality, of a SIRS classification, etc. In yet another alternative, a threshold that can provide an acceptable level of specificity and sensitivity in separating a population of stroke patients into "bins" such as a "first" subpopulation and a "second" subpopulation. A threshold value is selected to separate this first and second population by one or more of the following measures of test accuracy:

an odds ratio greater than 1, preferably at least about 2 or more or about 0.5 or less, more preferably at least about 3 or more or about 0.33 or less, still more preferably at least about 4 or more or about 0.25 or less, even more preferably at least about 5 or more or about 0.2 or less, and most preferably at least about 10 or more or about 0.1 or less;

a specificity of greater than 0.5, preferably at least about 0.6, more preferably at least about 0.7, still more preferably at least about 0.8, even more preferably at least about 0.9 and most preferably at least about 0.95, with a corresponding sensitivity greater than 0.2, preferably greater than about 0.3, more preferably greater than about 0.4, still more preferably at least about 0.5, even more preferably about 0.6, yet more preferably greater than about 0.7, still more preferably greater than about 0.8, more preferably greater than about 0.9, and most preferably greater than about 0.95;

a sensitivity of greater than 0.5, preferably at least about 0.6, more preferably at least about 0.7, still more preferably at least about 0.8, even more preferably at least about 0.9 and most preferably at least about 0.95, with a corresponding specificity greater than 0.2, preferably greater than about 0.3, more preferably greater than about 0.4, still more

preferably at least about 0.5, even more preferably about 0.6, yet more preferably greater than about 0.7, still more preferably greater than about 0.8, more preferably greater than about 0.9, and most preferably greater than about 0.95;

at least about 75% sensitivity, combined with at least about 75% specificity;

a positive likelihood ratio (calculated as sensitivity/(1-specificity)) of greater than 1, at least about 2, more preferably at least about 3, still more preferably at least about 5, and most preferably at least about 10; or

a negative likelihood ratio (calculated as (1-sensitivity)/specificity) of less than 1, less than or equal to about 0.5, more preferably less than or equal to about 0.3, and most preferably less than or equal to about 0.1.

[0028] The term "about" in the context of any of the above measurements refers to +/- 5% of a given measurement.

[0029] Multiple thresholds may also be used to assess a patient. For example, a "first" subpopulation identified by an existing disease, predisposition to a future outcome for the stroke patient, predisposition to mortality, etc., and a "second" subpopulation which is not so predisposed can be combined into a single group. This group is then subdivided into three or more equal parts (known as tertiles, quartiles, quintiles, etc., depending on the number of subdivisions). An odds ratio is assigned to stroke patients based on which subdivision they fall into. If one considers a tertile, the lowest or highest tertile can be used as a reference for comparison of the other subdivisions. This reference subdivision is assigned an odds ratio of 1. The second tertile is assigned an odds ratio that is relative to that first tertile. That is, someone in the second tertile might be 3 times more likely to suffer one or more future changes in disease status in comparison to someone in the first tertile. The third tertile is also assigned an odds ratio that is relative to that first tertile.

[0030] In certain embodiments, the assay method is an immunoassay. Antibodies for use in such assays will specifically bind a full length stroke biomarker of interest, and may also bind one or more polypeptides that are "related" thereto, as that term is defined hereinafter. Numerous immunoassay formats are known to those of skill in the art. Preferred body fluid samples are selected from the group consisting of urine, blood, serum, saliva, tears, and plasma.

[0031] The foregoing method steps should not be interpreted to mean that the stroke biomarker assay result(s) is/are used in isolation in the methods described herein. Rather,

additional variables or other clinical indicia may be included in the methods described herein. For example, a risk stratification, diagnostic, classification, monitoring, etc. method may combine the assay result(s) with one or more variables measured for the stroke patient selected from the group consisting of demographic information (e.g., weight, sex, age, race), medical history (e.g., family history, type of surgery, pre-existing disease such as aneurism, congestive heart failure, diabetes mellitus, hypertension, coronary artery disease, proteinuria, or renal insufficiency, clinical variables (e.g., blood pressure, temperature, respiration rate), risk scores (APACHE score, PREDICT score, TIMI Risk Score for UA/NSTEMI, Framingham Risk Score), Essen Stroke Risk Score, California risj score, ABCD score, or ABCD2 score. This list is not meant to be limiting.

[0032] When more than one marker is measured, the individual markers may be measured in samples obtained at the same time, or may be determined from samples obtained at different (e.g., an earlier or later) times. The individual markers may also be measured on the same or different body fluid samples. For example, one stroke biomarker may be measured in a serum or plasma sample and another stroke biomarker may be measured in a urine sample. In addition, assignment of a likelihood may combine an individual biomarker assay result with temporal changes in one or more additional variables.

[0033] In various related aspects, the present invention also relates to devices and kits for performing the methods described herein. Suitable kits comprise reagents sufficient for performing an assay for at least one of the described stroke biomarkers, together with instructions for performing the described threshold comparisons.

[0034] In certain embodiments, reagents for performing such assays are provided in an assay device, and such assay devices may be included in such a kit. Preferred reagents can comprise one or more solid phase antibodies, the solid phase antibody comprising antibody that detects the intended biomarker target(s) bound to a solid support. In the case of sandwich immunoassays, such reagents can also include one or more detectably labeled antibodies, the detectably labeled antibody comprising antibody that detects the intended biomarker target(s) bound to a detectable label. Additional optional elements that may be provided as part of an assay device are described hereinafter.

[0035] Detectable labels may include molecules that are themselves detectable (e.g., fluorescent moieties, electrochemical labels, ecl (electrochemical luminescence) labels,

metal chelates, colloidal metal particles, *etc.*) as well as molecules that may be indirectly detected by production of a detectable reaction product (*e.g.*, enzymes such as horseradish peroxidase, alkaline phosphatase, *etc.*) or through the use of a specific binding molecule which itself may be detectable (*e.g.*, a labeled antibody that binds to the second antibody, biotin, digoxigenin, maltose, oligohistidine, 2,4-dintrobenzene, phenylarsenate, ssDNA, dsDNA, etc.).

[0036] Generation of a signal from the signal development element can be performed using various optical, acoustical, and electrochemical methods well known in the art. Examples of detection modes include fluorescence, radiochemical detection, reflectance, absorbance, amperometry, conductance, impedance, interferometry, ellipsometry, *etc.* In certain of these methods, the solid phase antibody is coupled to a transducer (*e.g.*, a diffraction grating, electrochemical sensor, etc) for generation of a signal, while in others, a signal is generated by a transducer that is spatially separate from the solid phase antibody (*e.g.*, a fluorometer that employs an excitation light source and an optical detector). This list is not meant to be limiting. Antibody-based biosensors may also be employed to determine the presence or amount of analytes that optionally eliminate the need for a labeled molecule.

DETAILED DESCRIPTION OF THE INVENTION

[0037] The present invention relates to methods and compositions for diagnosis, differential diagnosis, risk stratification, monitoring, classifying and determination of treatment regimens in patients diagnosed with, or at risk of, stroke or other cerebral injury. In various embodiments, a measured concentration of one or more biomarkers selected from the group consisting of Agouti-related protein, Alpha-2 macroglobulin, Alpha-fetoprotein, Amphiregulin, Angiopoietin-1 receptor, Angiopoietin-related protein 3, Angiopoietin-related protein 4, Angiopoietin-related protein 6, Bone morphogenetic protein 7, Cadherin-3, Cancer Antigen 15-3, Cancer Antigen 19-9, Carcinoembryonic antigen-related cell adhesion molecule 5, C-C motif chemokine 1, C-C motif chemokine 13, C-C motif chemokine 15, C-C motif chemokine 17, C-C motif chemokine 19, C-C motif chemokine 26, C-C motif chemokine 8, Choriogonadotropin subunit beta, Clusterin, C-X-C motif chemokine 11, C-X-C motif chemokine 6, C-X-C motif chemokine 9, Cyclin-dependent kinase inhibitor 1, Endoglin, Epiregulin, Epithelial cell adhesion molecule, Erythropoietin, Fatty acid-binding protein, liver, Fibroblast growth

factor 19, Fibroblast growth factor 21, Fibroblast growth factor 23, Follistatin, Follitropin subunit beta, Galectin-3, Glial cell line-derived neurotrophic factor, Heat shock protein beta-1, Heparin-binding EGF-like growth factor, Heparin-binding growth factor 1, Hepatitis A virus cellular receptor 1, Hepatocyte growth factor receptor, Insulin receptor substrate 1, Insulin-like growth factor-binding protein 1, Insulin-like growth factor-binding protein 2, Insulin-like growth factor-binding protein 4, Intercellular adhesion molecule 3, Interleukin-1 receptor type I, Interleukin-1 receptor type II, Interleukin-11, Interleukin-21, Interleukin-28A, Interleukin-29, Interleukin-33, Interleukin-4 receptor alpha chain, Interleukin-6 receptor subunit alpha, Interleukin-6 receptor subunit beta, Keratin, type I cytoskeletal 19 (aa311-367), Kit ligand, Lutropin subunit beta, Lymphotactin, Macrophage colony-stimulating factor 1, Matrilysin, Metalloproteinase inhibitor 4, Growth-regulated alpha, beta, and gamma proteins (total), Mucin-16, Neprilysin, Neuronal cell adhesion molecule, NF-kappa-B inhibitor alpha, Osteocalcin, Oxidized low-density lipoprotein receptor 1, Parathyroid hormone, Platelet endothelial cell adhesion molecule, Probetacellulin, Pro-interleukin-16, Prolactin, Proprotein convertase subtilisin/kexin type 9, Prostate-specific antigen, Protein NOV homolog, Protransforming growth factor alpha, Serum amyloid P-component, Somatotropin, Stromal cell-derived factor 1, Thymic stromal lymphopoietin, Thyrotropin subunit beta, Transmembrane glycoprotein NMB, Tumor necrosis factor receptor superfamily member 8, Vascular endothelial growth factor D, Vascular endothelial growth factor receptor 1, Vascular endothelial growth factor receptor 2, Vascular endothelial growth factor receptor 3, and WAP four-disulfide core domain protein 2 or one or more markers related thereto, are correlated to the status of the patient. As described herein, measurement of one or more biomarkers of the present invention may be used, individually or in panels comprising a plurality of biomarkers, in methods and compositions for the diagnosis, prognosis, or differentiation of stroke or other cerebral injury in a subject. Such markers can be used in diagnosing and treating a subject and/or to monitor the course of a treatment regimen; for screening subjects for the occurrence or risk of a particular disease; and for screening compounds and pharmaceutical compositions that might provide a benefit in treating or preventing such conditions.

[0038] For purposes of this document, the following definitions apply:

[0039] The term “subject” as used herein refers to a human or non-human organism. Thus, the methods and compositions described herein are applicable to both human and

veterinary disease. Further, while a subject is preferably a living organism, the invention described herein may be used in post-mortem analysis as well. Preferred subjects are humans, and most preferably "patients," which as used herein refers to living humans that are receiving medical care for a disease or condition. This includes persons with no defined illness who are being investigated for signs of pathology. A "stroke patient" is a patient suffering from stroke. Stroke patients may be further classified as having a specific type of stroke (e. g., thrombotic, embolic, lacunar, hypoperfttsion, intracerebral hemorrhage, and sub arachnoid hemorrhage types of strokes. A "traumatic brain injury" patient is a patient for which an external force has traumatically injured the brain. Traumatic brain injury can be caused by a direct impact or by acceleration alone.

[0040] For purposes of this disclosure, the "acute" phase of a cerebral injury refers to up to 72 hours following the injury; "subacute" phase begins 3 days after the injury to 21 days after injury; and "chronic" phase begins after 21 days. For purposes of diagnosis, patients are preferably evaluated during the acute phase. For purposes of prognosis, patients may be evaluated during any of these three phases.

[0041] Specific neurologic dysfunctions or "stroke-associated symptoms" may include, but are not limited to, pain, headache, aphasia, apraxia, agnosia, amnesia, stupor, confusion, vertigo, coma, delirium, dementia, seizure, migraine insomnia, hypersomnia, sleep apnea, tremor, dyskinesia, paralysis, visual disturbances, diplopia, paresthesias, dysarthria, hemiplegia, hemianesthesia, hemianopia, etc. Patients exhibiting one or more of these symptoms but that have not suffered from a stroke are referred to herein as "stroke mimics".

[0042] Conditions within the differential diagnosis of stroke include brain tumor (including primary and metastatic disease), aneurysm, electrocution, bums, infections (e.g., meningitis), cerebral hypoxia, head injury (including concussion), stress, dehydration, nerve palsy (cranial or peripheral), hypoglycemia, migraine, multiple sclerosis, peripheral vascular disease, peripheral neuropathy, seizure (including grand mal seizure), subdural hematoma, syncope, and transient unilateral weakness. Preferred markers and marker panels are those that can distinguish stroke or traumatic brain injury from these stroke mimicking conditions.

[0043] Preferably, an analyte such as a stroke biomarker is measured in a sample. Such a sample may be obtained from a patient, such as a stroke patient. Preferred samples are body fluid samples.

[0044] The term "body fluid sample" as used herein refers to a sample of bodily fluid obtained for the purpose of diagnosis, prognosis, classification or evaluation of a stroke patient of interest, such as a patient or transplant donor. In certain embodiments, such a sample may be obtained for the purpose of determining the outcome of an ongoing condition or the effect of a treatment regimen on a condition. Preferred body fluid samples include blood, serum, plasma, cerebrospinal fluid, urine, saliva, sputum, and pleural effusions. In addition, one of skill in the art would realize that certain body fluid samples would be more readily analyzed following a fractionation or purification procedure, for example, separation of whole blood into serum or plasma components.

[0045] The term "diagnosis" as used herein refers to methods by which the skilled artisan can estimate and/or determine the probability ("a likelihood") of whether or not a patient is suffering from a given disease or condition. In the case of the present invention, "diagnosis" includes using the results of an assay, most preferably an immunoassay, for a stroke biomarker of the present invention, optionally together with other clinical characteristics, to arrive at a diagnosis (that is, the occurrence or nonoccurrence) of a disease or condition. That such a diagnosis is "determined" is not meant to imply that the diagnosis is 100% accurate. Many biomarkers are indicative of multiple conditions. The skilled clinician does not use biomarker results in an informational vacuum, but rather test results are used together with other clinical indicia to arrive at a diagnosis. Thus, a measured biomarker level on one side of a predetermined diagnostic threshold indicates a greater likelihood of the occurrence of disease in the stroke patient relative to a measured level on the other side of the predetermined diagnostic threshold.

[0046] Similarly, a prognostic risk signals a probability ("a likelihood") that a given course or outcome will occur. A level or a change in level of a prognostic indicator, which in turn is associated with an increased probability of morbidity or mortality is referred to as being "indicative of an increased likelihood" of an adverse outcome in a patient.

[0047] As used herein, the term "relating a signal to the presence or amount" of an analyte reflects the following understanding. Assay signals are typically related to the

presence or amount of an analyte through the use of a standard curve calculated using known concentrations of the analyte of interest. As the term is used herein, an assay is "configured to detect" an analyte if an assay can generate a detectable signal indicative of the presence or amount of a physiologically relevant concentration of the analyte. Because an antibody epitope is on the order of 8 amino acids, an immunoassay configured to detect a marker of interest will also detect polypeptides related to the marker sequence, so long as those polypeptides contain the epitope(s) necessary to bind to the antibody or antibodies used in the assay. The term "related marker" as used herein with regard to a biomarker such as one of the stroke biomarkers described herein refers to one or more fragments, variants, etc., of a particular marker or its biosynthetic parent that may be detected as a surrogate for the marker itself or as independent biomarkers. The term also refers to one or more polypeptides present in a biological sample that are derived from the biomarker precursor complexed to additional species, such as binding proteins, receptors, heparin, lipids, sugars, *etc.*

[0048] In this regard, the skilled artisan will understand that the signals obtained from an immunoassay are a direct result of complexes formed between one or more antibodies and the target biomolecule (*i.e.*, the analyte) and polypeptides containing the necessary epitope(s) to which the antibodies bind. While such assays may detect the full length biomarker and the assay result be expressed as a concentration of a biomarker of interest, the signal from the assay is actually a result of all such "immunoreactive" polypeptides present in the sample. Expression of biomarkers may also be determined by means other than immunoassays, including protein measurements (such as dot blots, western blots, chromatographic methods, mass spectrometry, *etc.*) and nucleic acid measurements (mRNA quatitation). This list is not meant to be limiting. With regard to biomarkers which exist in one form as type-I, type-II, or GPI-anchored membrane proteins, such membrane proteins typically comprise a substantial extracellular domain, some or all of which can be detected as soluble forms present in aqueous samples such as blood, serum, plasma, urine, etc., either as cleavage products or as splice variants which delete an effective membrane spanning domain. Preferred assays detect soluble forms of these biomarkers.

[0049] The term "positive going" marker as that term is used herein refer to a marker that is determined to be elevated in patients suffering from a disease or condition, relative to stroke not suffering from that disease or condition. The term "negative going" marker

as that term is used herein refer to a marker that is determined to be reduced in patients suffering from a disease or condition, relative to patients not suffering from that disease or condition.

[0050] Stroke Biomarkers

[0051] The following table provides a list of the biomarkers of the present invention, together with the Swiss-Prot entry number for the human precursor. As noted above, these biomarkers are referred to for convenience as "stroke biomarkers," although they may be used as described herein with regard to non-stroke conditions such as traumatic brain injury.

SwissProtNum	Preferred Name
O00253	Agouti-related protein
P01023	Alpha-2 macroglobulin
P02771	Alpha-fetoprotein
P15514	Amphiregulin
Q02763	Angiopoietin-1 receptor
Q9Y5C1	Angiopoietin-related protein 3
Q9BY76	Angiopoietin-related protein 4
Q8NI99	Angiopoietin-related protein 6
P18075	Bone morphogenetic protein 7
P22223	Cadherin-3
P15941	Cancer Antigen 15-3
na	Cancer Antigen 19-9
P06731	Carcinoembryonic antigen-related cell adhesion molecule 5
P22362	C-C motif chemokine 1
Q99616	C-C motif chemokine 13
Q16663	C-C motif chemokine 15
Q92583	C-C motif chemokine 17
Q99731	C-C motif chemokine 19
Q9Y258	C-C motif chemokine 26
P80075	C-C motif chemokine 8
P01233	Choriogonadotropin subunit beta
P10909	Clusterin
O14625	C-X-C motif chemokine 11
P80162	C-X-C motif chemokine 6
Q07325	C-X-C motif chemokine 9

P38936	Cyclin-dependent kinase inhibitor 1
P17813	Endoglin
O14944	Epiregulin
P16422	Epithelial cell adhesion molecule
P01588	Erythropoietin
P07148	Fatty acid-binding protein, liver
O95750	Fibroblast growth factor 19
Q9NSA1	Fibroblast growth factor 21
Q9GZV9	Fibroblast growth factor 23
P19883	Follistatin
P01225	Follitropin subunit beta
P17931	Galectin-3
P39905	Glial cell line-derived neurotrophic factor
P04792	Heat shock protein beta-1
Q99075	Heparin-binding EGF-like growth factor
P05230	Heparin-binding growth factor 1
Q96D42	Hepatitis A virus cellular receptor 1
P08581	Hepatocyte growth factor receptor
P35568	Insulin receptor substrate 1
P08833	Insulin-like growth factor-binding protein 1
P18065	Insulin-like growth factor-binding protein 2
P22692	Insulin-like growth factor-binding protein 4
P32942	Intercellular adhesion molecule 3
P14778	Interleukin-1 receptor type I
P27930	Interleukin-1 receptor type II
P20809	Interleukin-11
Q9HBE4	Interleukin-21
Q8IZJ0	Interleukin-28A
Q8IU54	Interleukin-29
O95760	Interleukin-33
P24394	Interleukin-4 receptor alpha chain
P08887	Interleukin-6 receptor subunit alpha
P40189	Interleukin-6 receptor subunit beta
P08727	Keratin, type I cytoskeletal 19 (aa311-367)
P21583	Kit ligand
P01229	Lutropin subunit beta
P47992	Lymphotactin
P09603	Macrophage colony-stimulating factor 1
P09237	Matrilysin

Q99727	Metalloproteinase inhibitor 4
P09341 P19875 P19876	Growth-regulated alpha, beta, and gamma proteins (total)
Q8WXI7	Mucin-16
P08473	Neprilysin
Q92823	Neuronal cell adhesion molecule
P25963	NF-kappa-B inhibitor alpha
P02818	Osteocalcin
P78380	Oxidized low-density lipoprotein receptor 1
P01270	Parathyroid hormone
P16284	Platelet endothelial cell adhesion molecule
P35070	Probetacellulin
Q14005	Pro-interleukin-16
P01236	Prolactin
Q8NBP7	Proprotein convertase subtilisin/kexin type 9
P07288	Prostate-specific antigen
P48745	Protein NOV homolog
P01135	Protransforming growth factor alpha
P02743	Serum amyloid P-component
P01241	Somatotropin
P48061	Stromal cell-derived factor 1
Q969D9	Thymic stromal lymphopoietin
P01222	Thyrotropin subunit beta
Q14956	Transmembrane glycoprotein NMB
P28908	Tumor necrosis factor receptor superfamily member 8
O43915	Vascular endothelial growth factor D
P17948	Vascular endothelial growth factor receptor 1
P35968	Vascular endothelial growth factor receptor 2
P35916	Vascular endothelial growth factor receptor 3
Q14508	WAP four-disulfide core domain protein 2

[0052] The following table provides an additional list of the biomarkers of the present invention, together with the Swiss-Prot entry number for the human precursor. As noted above, these biomarkers are referred to for convenience as “stroke biomarkers,” although they may be used as described herein with regard to non-stroke conditions such as traumatic brain injury.

Preferred Name	SwissProt Entry
Eotaxin	P51671
Epidermal growth factor receptor	P00533
S100A12	P80511
Metalloproteinase inhibitor 4 (TIMP-4)	Q99727
Prolactin	P01236

[0053] Marker Assays

[0054] In general, immunoassays involve contacting a sample containing or suspected of containing a biomarker of interest with at least one antibody that specifically binds to the biomarker. A signal is then generated indicative of the presence or amount of complexes formed by the binding of polypeptides in the sample to the antibody. The signal is then related to the presence or amount of the biomarker in the sample. Numerous methods and devices are well known to the skilled artisan for the detection and analysis of biomarkers. *See, e.g.*, U.S. Patents 6,143,576; 6,113,855; 6,019,944; 5,985,579; 5,947,124; 5,939,272; 5,922,615; 5,885,527; 5,851,776; 5,824,799; 5,679,526; 5,525,524; and 5,480,792, and *The Immunoassay Handbook*, David Wild, ed. Stockton Press, New York, 1994, each of which is hereby incorporated by reference in its entirety, including all tables, figures and claims.

[0055] The assay devices and methods known in the art can utilize labeled molecules in various sandwich, competitive, or non-competitive assay formats, to generate a signal that is related to the presence or amount of the biomarker of interest. Suitable assay formats also include chromatographic, mass spectrographic, and protein "blotting" methods. Additionally, certain methods and devices, such as biosensors and optical immunoassays, may be employed to determine the presence or amount of analytes without the need for a labeled molecule. *See, e.g.*, U.S. Patents 5,631,171; and 5,955,377, each of which is hereby incorporated by reference in its entirety, including all tables, figures and claims. One skilled in the art also recognizes that robotic instrumentation including but not limited to Beckman ACCESS®, Abbott AXSYM®, Roche ELECSYS®, Dade Behring STRATUS® systems are among the immunoassay analyzers that are capable of performing immunoassays. But any suitable immunoassay may be

utilized, for example, enzyme-linked immunoassays (ELISA), radioimmunoassays (RIAs), competitive binding assays, and the like.

[0056] Antibodies or other polypeptides may be immobilized onto a variety of solid supports for use in assays. Solid phases that may be used to immobilize specific binding members include include those developed and/or used as solid phases in solid phase binding assays. Examples of suitable solid phases include membrane filters, cellulose-based papers, beads (including polymeric, latex and paramagnetic particles), glass, silicon wafers, microparticles, nanoparticles, TentaGels, AgroGels, PEGA gels, SPOCC gels, and multiple-well plates. An assay strip could be prepared by coating the antibody or a plurality of antibodies in an array on solid support. This strip could then be dipped into the test sample and then processed quickly through washes and detection steps to generate a measurable signal, such as a colored spot. Antibodies or other polypeptides may be bound to specific zones of assay devices either by conjugating directly to an assay device surface, or by indirect binding. In an example of the later case, antibodies or other polypeptides may be immobilized on particles or other solid supports, and that solid support immobilized to the device surface.

[0057] Biological assays require methods for detection, and one of the most common methods for quantitation of results is to conjugate a detectable label to a protein or nucleic acid that has affinity for one of the components in the biological system being studied. Detectable labels may include molecules that are themselves detectable (*e.g.*, fluorescent moieties, electrochemical labels, metal chelates, *etc.*) as well as molecules that may be indirectly detected by production of a detectable reaction product (*e.g.*, enzymes such as horseradish peroxidase, alkaline phosphatase, *etc.*) or by a specific binding molecule which itself may be detectable (e.g., biotin, digoxigenin, maltose, oligohistidine, 2,4-dintrobenzene, phenylarsenate, ssDNA, dsDNA, etc.).

[0058] Preparation of solid phases and detectable label conjugates often comprise the use of chemical cross-linkers. Cross-linking reagents contain at least two reactive groups, and are divided generally into homofunctional cross-linkers (containing identical reactive groups) and heterofunctional cross-linkers (containing non-identical reactive groups). Homobifunctional cross-linkers that couple through amines, sulfhydryls or react non-specifically are available from many commercial sources. Maleimides, alkyl and aryl halides, alpha-haloacyls and pyridyl disulfides are thiol reactive groups. Maleimides, alkyl and aryl halides, and alpha-haloacyls react with sulfhydryls to form thiol ether

bonds, while pyridyl disulfides react with sulfhydryls to produce mixed disulfides. The pyridyl disulfide product is cleavable. Imidoesters are also very useful for protein-protein cross-links. A variety of heterobifunctional cross-linkers, each combining different attributes for successful conjugation, are commercially available.

[0059] In certain aspects, the present invention provides kits for the analysis of the described stroke biomarkers. The kit comprises reagents for the analysis of at least one test sample which comprise at least one antibody that binds a stroke biomarker. The kit can also include devices and instructions for performing one or more of the diagnostic and/or prognostic correlations described herein. Preferred kits will comprise an antibody pair for performing a sandwich assay, or a labeled species for performing a competitive assay, for the analyte. Preferably, an antibody pair comprises a first antibody conjugated to a solid phase and a second antibody conjugated to a detectable label, wherein each of the first and second antibodies bind a stroke biomarker. Most preferably each of the antibodies are monoclonal antibodies. The instructions for use of the kit and performing the correlations can be in the form of labeling, which refers to any written or recorded material that is attached to, or otherwise accompanies a kit at any time during its manufacture, transport, sale or use. For example, the term labeling encompasses advertising leaflets and brochures, packaging materials, instructions, audio or video cassettes, computer discs, as well as writing imprinted directly on kits.

[0060] Antibodies

[0061] The term “antibody” as used herein refers to a peptide or polypeptide derived from, modeled after or substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof, capable of specifically binding an antigen or epitope. See, e.g. Fundamental Immunology, 3rd Edition, W.E. Paul, ed., Raven Press, N.Y. (1993); Wilson (1994; J. Immunol. Methods 175:267-273; Yarmush (1992) J. Biochem. Biophys. Methods 25:85-97. The term antibody includes antigen-binding portions, i.e., "antigen binding sites," (e.g., fragments, subsequences, complementarity determining regions (CDRs)) that retain capacity to bind antigen, including (i) a Fab fragment, a monovalent fragment consisting of the VL, VH, CL and CHl domains; (ii) a F(ab')2 fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the VH and CHl domains; (iv) a Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (v) a dAb fragment (Ward et al., (1989) Nature 341:544-546), which consists of a VH domain;

and (vi) an isolated complementarity determining region (CDR). Single chain antibodies are also included by reference in the term "antibody."

[0062] Antibodies used in the immunoassays described herein preferably specifically bind to a stroke biomarker of the present invention. The term “specifically binds” is not intended to indicate that an antibody binds exclusively to its intended target since, as noted above, an antibody binds to any polypeptide displaying the epitope(s) to which the antibody binds. Rather, an antibody “specifically binds” if its affinity for its intended target is about 5-fold greater when compared to its affinity for a non-target molecule which does not display the appropriate epitope(s). Preferably the affinity of the antibody will be at least about 5 fold, preferably 10 fold, more preferably 25-fold, even more preferably 50-fold, and most preferably 100-fold or more, greater for a target molecule than its affinity for a non-target molecule. In preferred embodiments, Preferred antibodies bind with affinities of at least about 10^7 M^{-1}, and preferably between about 10^8 M^{-1} to about 10^9 M^{-1}, about 10^9 M^{-1} to about 10^{10} M^{-1}, or about 10^{10} M^{-1} to about 10^{12} M^{-1}.

[0063] Affinity is calculated as $K_d = k_{on}/k_{off}$ (k_{off} is the dissociation rate constant, K_{on} is the association rate constant and K_d is the equilibrium constant). Affinity can be determined at equilibrium by measuring the fraction bound (r) of labeled ligand at various concentrations (c). The data are graphed using the Scatchard equation: r/c = K(n-r): where r = moles of bound ligand/mole of receptor at equilibrium; c = free ligand concentration at equilibrium; K = equilibrium association constant; and n = number of ligand binding sites per receptor molecule. By graphical analysis, r/c is plotted on the Y-axis versus r on the X-axis, thus producing a Scatchard plot. Antibody affinity measurement by Scatchard analysis is well known in the art. *See, e.g.*, van Erp *et al., J. Immunoassay* 12: 425-43, 1991; Nelson and Griswold, *Comput. Methods Programs Biomed.* 27: 65-8, 1988.

[0064] The term “epitope” refers to an antigenic determinant capable of specific binding to an antibody. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and nonconformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents.

[0065] Numerous publications discuss the use of phage display technology to produce and screen libraries of polypeptides for binding to a selected analyte. *See, e.g*, Cwirla *et*

al., Proc. Natl. Acad. Sci. USA 87, 6378-82, 1990; Devlin *et al., Science* 249, 404-6, 1990, Scott and Smith, *Science* 249, 386-88, 1990; and Ladner *et al.*, U.S. Pat. No. 5,571,698. A basic concept of phage display methods is the establishment of a physical association between DNA encoding a polypeptide to be screened and the polypeptide. This physical association is provided by the phage particle, which displays a polypeptide as part of a capsid enclosing the phage genome which encodes the polypeptide. The establishment of a physical association between polypeptides and their genetic material allows simultaneous mass screening of very large numbers of phage bearing different polypeptides. Phage displaying a polypeptide with affinity to a target bind to the target and these phage are enriched by affinity screening to the target. The identity of polypeptides displayed from these phage can be determined from their respective genomes. Using these methods a polypeptide identified as having a binding affinity for a desired target can then be synthesized in bulk by conventional means. *See, e.g.*, U.S. Patent No. 6,057,098, which is hereby incorporated in its entirety, including all tables, figures, and claims.

[0066] The antibodies that are generated by these methods may then be selected by first screening for affinity and specificity with the purified polypeptide of interest and, if required, comparing the results to the affinity and specificity of the antibodies with polypeptides that are desired to be excluded from binding. The screening procedure can involve immobilization of the purified polypeptides in separate wells of microtiter plates. The solution containing a potential antibody or groups of antibodies is then placed into the respective microtiter wells and incubated for about 30 min to 2 h. The microtiter wells are then washed and a labeled secondary antibody (for example, an anti-mouse antibody conjugated to alkaline phosphatase if the raised antibodies are mouse antibodies) is added to the wells and incubated for about 30 min and then washed. Substrate is added to the wells and a color reaction will appear where antibody to the immobilized polypeptide(s) are present.

[0067] The antibodies so identified may then be further analyzed for affinity and specificity in the assay design selected. In the development of immunoassays for a target protein, the purified target protein acts as a standard with which to judge the sensitivity and specificity of the immunoassay using the antibodies that have been selected. Because the binding affinity of various antibodies may differ; certain antibody pairs (e.g., in sandwich assays) may interfere with one another sterically, etc., assay performance of an

antibody may be a more important measure than absolute affinity and specificity of an antibody.

[0068] While the present application describes antibody-based binding assays in detail, alternatives to antibodies as binding species in assays are well known in the art. These include receptors for a particular target, aptamers, etc. Aptamers are oligonucleic acid or peptide molecules that bind to a specific target molecule. Aptamers are usually created by selecting them from a large random sequence pool, but natural aptamers also exist. High-affinity aptamers containing modified nucleotides can confer improved characteristics on the ligand, such as improved in vivo stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions, and may include amino acid side chain functionalities.

[0069] Assay Correlations

[0070] The term "correlating" as used herein in reference to the use of biomarkers refers to comparing the presence or amount of the biomarker(s) in a patient to its presence or amount in persons known to suffer from, or known to be at risk of, a given condition; or in persons known to be free of a given condition. Often, this takes the form of comparing an assay result in the form of a biomarker concentration to a predetermined threshold selected to be indicative of the occurrence or nonoccurrence of a disease or the likelihood of some future outcome.

[0071] Selecting a diagnostic threshold involves, among other things, consideration of the probability of disease, distribution of true and false diagnoses at different test thresholds, and estimates of the consequences of treatment (or a failure to treat) based on the diagnosis. For example, when considering administering a specific therapy which is highly efficacious and has a low level of risk, few tests are needed because clinicians can accept substantial diagnostic uncertainty. On the other hand, in situations where treatment options are less effective and more risky, clinicians often need a higher degree of diagnostic certainty. Thus, cost/benefit analysis is involved in selecting a diagnostic threshold.

[0072] Suitable thresholds may be determined in a variety of ways. For example, one recommended diagnostic threshold for the diagnosis of acute myocardial infarction using cardiac troponin is the 97.5th percentile of the concentration seen in a normal population.

Another method may be to look at serial samples from the same patient, where a prior "baseline" result is used to monitor for temporal changes in a biomarker level.

[0073] Population studies may also be used to select a decision threshold. Reciever Operating Characteristic ("ROC") arose from the field of signal dectection therory developed during World War II for the analysis of radar images, and ROC analysis is often used to select a threshold able to best distinguish a "diseased" subpopulation from a "nondiseased" subpopulation. A false positive in this case occurs when the person tests positive, but actually does not have the disease. A false negative, on the other hand, occurs when the person tests negative, suggesting they are healthy, when they actually do have the disease. To draw a ROC curve, the true positive rate (TPR) and false positive rate (FPR) are determined as the decision threshold is varied continuously. Since TPR is equivalent with sensitivity and FPR is equal to 1 - specificity, the ROC graph is sometimes called the sensitivity vs (1 - specificity) plot. A perfect test will have an area under the ROC curve of 1.0; a random test will have an area of 0.5. A threshold is selected to provide an acceptable level of specificity and sensitivity.

[0074] In this context, "diseased" is meant to refer to a population having one characteristic (the presence of a disease or condition or the occurrence of some outcome) and "nondiseased" is meant to refer to a population lacking the characteristic. While a single decision threshold is the simplest application of such a method, multiple decision thresholds may be used. For example, below a first threshold, the absence of disease may be assigned with relatively high confidence, and above a second threshold the presence of disease may also be assigned with relatively high confidence. Between the two thresholds may be considered indeterminate. This is meant to be exemplary in nature only.

[0075] In addition to threshold comparisons, other methods for correlating assay results to a patient classification (occurrence or nonoccurrence of disease, likelihood of an outcome, etc.) include decision trees, rule sets, Bayesian methods, and neural network methods. These methods can produce probability values representing the degree to which a patient belongs to one classification out of a plurality of classifications.

[0076] Measures of test accuracy may be obtained as described in Fischer *et al., Intensive Care Med.* 29: 1043-51, 2003, and used to determine the effectiveness of a given biomarker. These measures include sensitivity and specificity, predictive values, likelihood ratios, diagnostic odds ratios, and ROC curve areas. The area under the curve

("AUC") of a ROC plot is equal to the probability that a classifier will rank a randomly chosen positive instance higher than a randomly chosen negative one. The area under the ROC curve may be thought of as equivalent to the Mann-Whitney U test, which tests for the median difference between scores obtained in the two groups considered if the groups are of continuous data, or to the Wilcoxon test of ranks.

[0077] As discussed above, suitable tests may exhibit one or more of the following results on these various measures: a specificity of greater than 0.5, preferably at least 0.6, more preferably at least 0.7, still more preferably at least 0.8, even more preferably at least 0.9 and most preferably at least 0.95, with a corresponding sensitivity greater than 0.2, preferably greater than 0.3, more preferably greater than 0.4, still more preferably at least 0.5, even more preferably 0.6, yet more preferably greater than 0.7, still more preferably greater than 0.8, more preferably greater than 0.9, and most preferably greater than 0.95; a sensitivity of greater than 0.5, preferably at least 0.6, more preferably at least 0.7, still more preferably at least 0.8, even more preferably at least 0.9 and most preferably at least 0.95, with a corresponding specificity greater than 0.2, preferably greater than 0.3, more preferably greater than 0.4, still more preferably at least 0.5, even more preferably 0.6, yet more preferably greater than 0.7, still more preferably greater than 0.8, more preferably greater than 0.9, and most preferably greater than 0.95; at least 75% sensitivity, combined with at least 75% specificity; a ROC curve area of greater than 0.5, preferably at least 0.6, more preferably 0.7, still more preferably at least 0.8, even more preferably at least 0.9, and most preferably at least 0.95; an odds ratio different from 1, preferably at least about 2 or more or about 0.5 or less, more preferably at least about 3 or more or about 0.33 or less, still more preferably at least about 4 or more or about 0.25 or less, even more preferably at least about 5 or more or about 0.2 or less, and most preferably at least about 10 or more or about 0.1 or less; a positive likelihood ratio (calculated as sensitivity/(1-specificity)) of greater than 1, at least 2, more preferably at least 3, still more preferably at least 5, and most preferably at least 10; and or a negative likelihood ratio (calculated as (1-sensitivity)/specificity) of less than 1, less than or equal to 0.5, more preferably less than or equal to 0.3, and most preferably less than or equal to 0.1

[0078] Additional clinical indicia may be combined with the stroke biomarker assay result(s) of the present invention. Other clinical indicia which may be combined with the stroke biomarker assay result(s) of the present invention includes demographic

information (e.g., weight, sex, age, race), medical history (e.g., family history, type of surgery, pre-existing disease such as aneurism, congestive heart failure, preeclampsia, eclampsia, diabetes mellitus, hypertension, coronary artery disease, proteinuria, or renal insufficiency), risk scores (APACHE score, PREDICT score, TIMI Risk Score for UA/NSTEMI, Framingham Risk Score), etc.

[0079] Combining assay results/clinical indicia in this manner can comprise the use of multivariate logistical regression, loglinear modeling, neural network analysis, n-of-m analysis, decision tree analysis, etc. This list is not meant to be limiting.

[0080] Selecting a Treatment Regimen

[0081] Once a diagnosis is obtained, the clinician can readily select a treatment regimen that is compatible with the diagnosis. The skilled artisan is aware of appropriate treatments for numerous diseases discussed in relation to the methods of diagnosis described herein. See, e.g., Merck Manual of Diagnosis and Therapy, 17th Ed. Merck Research Laboratories, Whitehouse Station, NJ, 1999. In addition, since the methods and compositions described herein provide prognostic information, the markers of the present invention may be used to monitor a course of treatment. For example, improved or worsened prognostic state may indicate that a particular treatment is or is not efficacious.

[0082] One skilled in the art readily appreciates that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The examples provided herein are representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention.

[0083] Example 1. Immunoassay format

[0084] Analytes are measured using standard sandwich enzyme immunoassay techniques. A first antibody which binds the analyte is immobilized in wells of a 96 well polystyrene microplate. Analyte standards and test samples are pipetted into the appropriate wells and any analyte present is bound by the immobilized antibody. After washing away any unbound substances, a horseradish peroxidase-conjugated second antibody which binds the analyte is added to the wells, thereby forming sandwich complexes with the analyte (if present) and the first antibody. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution comprising tetramethylbenzidine and hydrogen peroxide is added to the wells. Color develops in

proportion to the amount of analyte present in the sample. The color development is stopped and the intensity of the color is measured at 540 nm or 570 nm. An analyte concentration is assigned to the test sample by comparison to a standard curve determined from the analyte standards.

[0085] A list of the markers tested, with the associated Swiss-Prot entry number for the marker precursor and the units of measurement, is provided in the following table:

[0086] Table 1:

O00253	Agouti-related protein	ng/ml
P01023	Alpha-2 macroglobulin	mg/mL
P02771	Alpha-fetoprotein	ng/mL
P15514	Amphiregulin	pg/ml
Q02763	Angiopoietin-1 receptor	ng/ml
Q9Y5C1	Angiopoietin-related protein 3	ng/ml
Q9BY76	Angiopoietin-related protein 4	ng/ml
Q8NI99	Angiopoietin-related protein 6	ng/ml
P18075	Bone morphogenetic protein 7	pg/ml
P22223	Cadherin-3	ng/ml
P15941	Cancer Antigen 15-3	U/ml
na	Cancer Antigen 19-9	U/mL
P06731	Carcinoembryonic antigen-related cell adhesion molecule 5	ng/mL
P22362	C-C motif chemokine 1	pg/ml
Q99616	C-C motif chemokine 13	pg/ml
Q16663	C-C motif chemokine 15	pg/ml
Q92583	C-C motif chemokine 17	pg/ml
Q99731	C-C motif chemokine 19	pg/ml
Q9Y258	C-C motif chemokine 26	pg/ml
P80075	C-C motif chemokine 8	pg/ml
P01233	Choriogonadotropin subunit beta	mU/ml
P10909	Clusterin	ng/mL
O14625	C-X-C motif chemokine 11	pg/ml
P80162	C-X-C motif chemokine 6	pg/ml
Q07325	C-X-C motif chemokine 9	pg/ml
P38936	Cyclin-dependent kinase inhibitor 1	pg/ml
P17813	Endoglin	ng/ml
O14944	Epiregulin	pg/ml
P16422	Epithelial cell adhesion molecule	pg/ml

P01588	Erythropoietin	pg/mL
P07148	Fatty acid-binding protein, liver	ng/ml
O95750	Fibroblast growth factor 19	ng/ml
Q9NSA1	Fibroblast growth factor 21	ng/ml
Q9GZV9	Fibroblast growth factor 23	ng/ml
P19883	Follistatin	pg/ml
P01225	Follitropin subunit beta	mIU/mL
P17931	Galectin-3	ng/ml
P39905	Glial cell line-derived neurotrophic factor	pg/ml
P04792	Heat shock protein beta-1	ng/ml
Q99075	Heparin-binding EGF-like growth factor	pg/ml
P05230	Heparin-binding growth factor 1	pg/ml
Q96D42	Hepatitis A virus cellular receptor 1	pg/ml
P08581	Hepatocyte growth factor receptor	pg/ml
P35568	Insulin receptor substrate 1	ng/ml
P08833	Insulin-like growth factor-binding protein 1	ng/ml
P18065	Insulin-like growth factor-binding protein 2	ng/ml
P22692	Insulin-like growth factor-binding protein 4	ng/ml
P32942	Intercellular adhesion molecule 3	ng/ml
P14778	Interleukin-1 receptor type I	pg/mL
P27930	Interleukin-1 receptor type II	pg/ml
P20809	Interleukin-11	pg/ml
Q9HBE4	Interleukin-21	pg/ml
Q8IZJ0	Interleukin-28A	pg/ml
Q8IU54	Interleukin-29	pg/ml
O95760	Interleukin-33	pg/ml
P24394	Interleukin-4 receptor alpha chain	pg/ml
P08887	Interleukin-6 receptor subunit alpha	pg/ml
P40189	Interleukin-6 receptor subunit beta	pg/ml
P08727	Keratin, type I cytoskeletal 19 (aa311-367)	pg/ml
P21583	Kit ligand	pg/mL
P01229	Lutropin subunit beta	mIU/mL
P47992	Lymphotactin	ng/mL
P09603	Macrophage colony-stimulating factor 1	pg/ml
P09237	Matrilysin	pg/ml
Q99727	Metalloproteinase inhibitor 4	pg/ml
P09341 P19875 P19876	Growth-regulated alpha, beta, and gamma proteins (total)	pg/ml
Q8WXI7	Mucin-16	U/mL

P08473	Neprilysin	ng/ml
Q92823	Neuronal cell adhesion molecule	pg/ml
P25963	NF-kappa-B inhibitor alpha	ng/ml
P02818	Osteocalcin	pg/ml
P78380	Oxidized low-density lipoprotein receptor 1	pg/ml
P01270	Parathyroid hormone	pg/ml
P16284	Platelet endothelial cell adhesion molecule	ng/ml
P35070	Probetacellulin	pg/ml
Q14005	Pro-interleukin-16	pg/mL
P01236	Prolactin	ng/ml
Q8NBP7	Proprotein convertase subtilisin/kexin type 9	pg/ml
P07288	Prostate-specific antigen	ng/mL
P48745	Protein NOV homolog	pg/ml
P01135	Protransforming growth factor alpha	pg/ml
P02743	Serum amyloid P-component	ug/mL
P01241	Somatotropin	ng/mL
P48061	Stromal cell-derived factor 1	pg/ml
Q969D9	Thymic stromal lymphopoietin	pg/ml
P01222	Thyrotropin subunit beta	uIU/ml
Q14956	Transmembrane glycoprotein NMB	ng/ml
P28908	Tumor necrosis factor receptor superfamily member 8	pg/ml
O43915	Vascular endothelial growth factor D	pg/ml
P17948	Vascular endothelial growth factor receptor 1	pg/ml
P35968	Vascular endothelial growth factor receptor 2	pg/ml
P35916	Vascular endothelial growth factor receptor 3	pg/ml
Q14508	WAP four-disulfide core domain protein 2	pg/ml

[0087] Example 2. Use of analyte as a marker for assessing patients for stroke and/or TIA

[0088] Patients from the emergency department (ED) were classified as stroke mimics, positive for transient ischemic attack, positive for ischemic stroke, or positive for hemorrhagic stroke, in each case according to clinical diagnosis at the study site.

[0089] Two cohorts were defined as Cohort 1 and Cohort 2 as described in the following data tables. Plasma samples from each patient in Cohorts 1 and 2 were collected at enrollment. The concentrations of the analyte in these samples were measured by standard immunoassay methods using commercially available assay reagents. A receiver operating characteristic (ROC) curve was generated using the

concentrations, and the performance of the analyte is assessed by the area under the ROC curve (AUC). The two-tailed *p*-value of the AUC for the analyte was also calculated to determine statistical significance. "Inc/Dec" indicates if the marker is increasing or decreasing in Cohort 1 relative to Cohort 2.

[0090] Table 2: Ischemic stroke + TIA (Cohort 1) vs. Mimics (Cohort 2)

Marker Name	p	Inc/Dec	AUC
Agouti-related protein	0.057	Inc	0.538
Alpha-2 macroglobulin	0.0502	Dec	0.663
Alpha-fetoprotein	0.0583	Dec	0.518
Amphiregulin	0.056	Dec	0.51
Angiopoietin-1 receptor	0.0559	Inc	0.509
Angiopoietin-related protein 3	0.0578	Dec	0.511
Angiopoietin-related protein 4	0.0568	Inc	0.548
Angiopoietin-related protein 6	0.0528	Dec	0.637
Bone morphogenetic protein 7	0.0557	Dec	0.521
Cadherin-3	0.0549	Dec	0.549
Cancer Antigen 15-3	0.052	Dec	0.659
Cancer Antigen 19-9	0.0565	Dec	0.574
Carcinoembryonic antigen-related cell adhesion molecule 5	0.0578	Inc	0.534
C-C motif chemokine 1	0.0631	Inc	0.552
C-C motif chemokine 13	0.0459	Dec	0.725
C-C motif chemokine 15	0.0617	Dec	0.598
C-C motif chemokine 17	0.0516	Dec	0.64
C-C motif chemokine 19	0.0548	Dec	0.597
C-C motif chemokine 26	0.0634	Dec	0.543
C-C motif chemokine 8	0.064	Dec	0.508
Choriogonadotropin subunit beta	0.0522	Inc	0.656
Clusterin	0.056	Dec	0.502
C-X-C motif chemokine 11	0.0577	Dec	0.513
C-X-C motif chemokine 6	0.0579	Dec	0.503
C-X-C motif chemokine 9	0.0573	Dec	0.529
Cyclin-dependent kinase inhibitor 1	0.0496	Inc	0.665
Endoglin	0.056	Inc	0.501
Epiregulin	0.0547	Dec	0.561
Epithelial cell adhesion molecule	0.0556	Dec	0.524
Erythropoietin	0.0551	Inc	0.542
Fatty acid-binding protein, liver	0.0575	Dec	0.524

Fibroblast growth factor 19	0.0579	Dec	0.508
Fibroblast growth factor 21	0.0571	Dec	0.538
Fibroblast growth factor 23	0.0568	Dec	0.547
Follistatin	0.0467	Inc	0.707
Follitropin subunit beta	0.055	Dec	0.593
Galectin-3	0.0553	Dec	0.535
Glial cell line-derived neurotrophic factor	0.0636	Dec	0.514
Heat shock protein beta-1	0.0532	Inc	0.597
Heparin-binding EGF-like growth factor	0.056	Dec	0.508
Heparin-binding growth factor 1	0.0555	Inc	0.528
Hepatitis A virus cellular receptor 1	0.0559	Dec	0.51
Hepatocyte growth factor receptor	0.0541	Dec	0.573
Insulin receptor substrate 1	0.0501	Inc	0.656
Insulin-like growth factor-binding protein 1	0.056	Inc	0.506
Insulin-like growth factor-binding protein 2	0.0537	Inc	0.583
Insulin-like growth factor-binding protein 4	0.0557	Inc	0.519
Intercellular adhesion molecule 3	0.0514	Dec	0.658
Interleukin-1 receptor type I	0.0512	Dec	0.663
Interleukin-1 receptor type II	0.0557	Dec	0.576
Interleukin-11	0.0573	Inc	0.529
Interleukin-21	0.0633	Inc	0.546
Interleukin-28A	0.0634	Dec	0.54
Interleukin-29	0.0576	Inc	0.513
Interleukin-33	0.064	Dec	0.501
Interleukin-4 receptor alpha chain	0.0534	Inc	0.625
Interleukin-6 receptor subunit alpha	0.0531	Dec	0.63
Interleukin-6 receptor subunit beta	0.0517	Dec	0.654
Keratin, type I cytoskeletal 19 (aa311-367)	0.0562	Inc	0.58
Kit ligand	0.0619	Dec	0.591
Lutropin subunit beta	0.0539	Dec	0.616
Lymphotactin	0.0555	Inc	0.581
Macrophage colony-stimulating factor 1	0.0573	Dec	0.527
Matrilysin	0.0558	Inc	0.52
Metalloproteinase inhibitor 4	0.0566	Inc	0.538
Mix of Growth-regulated alpha, beta, and gamma proteins	0.063	Inc	0.537
Mucin-16	0.0575	Inc	0.544
Neprilysin	0.0549	Inc	0.551
Neuronal cell adhesion molecule	0.0543	Dec	0.569
NF-kappa-B inhibitor alpha	0.0538	Inc	0.581

Osteocalcin	0.0565	Dec	0.556
Oxidized low-density lipoprotein receptor 1	0.0633	Dec	0.515
Parathyroid hormone	0.057	Dec	0.537
Platelet endothelial cell adhesion molecule	0.0535	Inc	0.59
Probetacellulin	0.055	Dec	0.548
Pro-interleukin-16	0.0624	Dec	0.578
Prolactin	0.0539	Dec	0.629
Proprotein convertase subtilisin/kexin type 9	0.0554	Dec	0.534
Prostate-specific antigen	0.0559	Inc	0.588
Protein NOV homolog	0.0561	Inc	0.501
Protransforming growth factor alpha	0.0542	Dec	0.572
Serum amyloid P-component	0.0509	Dec	0.652
Somatotropin	0.0562	Inc	0.564
Stromal cell-derived factor 1	0.0616	Dec	0.6
Thymic stromal lymphopoietin	0.064	Dec	0.507
Thyrotropin subunit beta	0.0569	Dec	0.543
Transmembrane glycoprotein NMB	0.0536	Dec	0.586
Tumor necrosis factor receptor superfamily member 8	0.0569	Dec	0.541
Vascular endothelial growth factor D	0.0529	Dec	0.604
Vascular endothelial growth factor receptor 1	0.0529	Dec	0.635
Vascular endothelial growth factor receptor 2	0.0495	Dec	0.688
Vascular endothelial growth factor receptor 3	0.0552	Dec	0.589
WAP four-disulfide core domain protein 2	0.0533	Inc	0.638

[0091] Table 3: Ischemic stroke (Cohort 1) vs. Mimics + TIA (Cohort 2)

Agouti-related protein	0.9584	Dec	0.503
Alpha-2 macroglobulin	0.0088	Dec	0.631
Alpha-fetoprotein	0.3801	Inc	0.545
Amphiregulin	0.3556	Inc	0.547
Angiopoietin-1 receptor	0.7045	Inc	0.519
Angiopoietin-related protein 3	0.8477	Inc	0.51
Angiopoietin-related protein 4	0.0435	Inc	0.602
Angiopoietin-related protein 6	0.0822	Dec	0.589
Bone morphogenetic protein 7	0.4954	Inc	0.535
Cadherin-3	0.3187	Dec	0.551
Cancer Antigen 15-3	0.0373	Dec	0.606
Cancer Antigen 19-9	0.552	Dec	0.531
Carcinoembryonic antigen-related cell adhesion molecule 5	0.7858	Dec	0.514

C-C motif chemokine 1	0.3197	Inc	0.567
C-C motif chemokine 13	0.0019	Dec	0.654
C-C motif chemokine 15	0.8491	Dec	0.513
C-C motif chemokine 17	0.0015	Dec	0.657
C-C motif chemokine 19	0.1684	Dec	0.57
C-C motif chemokine 26	0.627	Dec	0.533
C-C motif chemokine 8	0.974	Dec	0.502
Choriogonadotropin subunit beta	0.0442	Inc	0.602
Clusterin	0.5426	Inc	0.531
C-X-C motif chemokine 11	0.6491	Inc	0.523
C-X-C motif chemokine 6	0.3822	Inc	0.545
C-X-C motif chemokine 9	0.6153	Dec	0.526
Cyclin-dependent kinase inhibitor 1	0.0294	Inc	0.608
Endoglin	0.854	Dec	0.509
Epiregulin	0.8199	Inc	0.512
Epithelial cell adhesion molecule	0.5701	Dec	0.529
Erythropoietin	0.7817	Dec	0.514
Fatty acid-binding protein, liver	0.7862	Dec	0.514
Fibroblast growth factor 19	0.638	Inc	0.524
Fibroblast growth factor 21	0.8553	Inc	0.509
Fibroblast growth factor 23	0.2176	Dec	0.563
Follistatin	0.0075	Inc	0.632
Follitropin subunit beta	0.7616	Dec	0.516
Galectin-3	0.5639	Inc	0.529
Glial cell line-derived neurotrophic factor	0.4663	Dec	0.55
Heat shock protein beta-1	0.101	Inc	0.583
Heparin-binding EGF-like growth factor	0.135	Inc	0.576
Heparin-binding growth factor 1	0.7129	Inc	0.519
Hepatitis A virus cellular receptor 1	0.9059	Dec	0.506
Hepatocyte growth factor receptor	0.7402	Dec	0.517
Insulin receptor substrate 1	0.1939	Inc	0.566
Insulin-like growth factor-binding protein 1	0.6237	Inc	0.525
Insulin-like growth factor-binding protein 2	0.0247	Inc	0.612
Insulin-like growth factor-binding protein 4	0.6447	Inc	0.523
Intercellular adhesion molecule 3	0.1986	Dec	0.568
Interleukin-1 receptor type I	0.0454	Dec	0.601
Interleukin-1 receptor type II	0.1684	Dec	0.57
Interleukin-11	0.1752	Inc	0.569
Interleukin-21	0.1986	Dec	0.587

Interleukin-28A	0.0646	Dec	0.624
Interleukin-29	0.7652	Dec	0.515
Interleukin-33	0.6635	Dec	0.53
Interleukin-4 receptor alpha chain	0.1325	Inc	0.577
Interleukin-6 receptor subunit alpha	0.1357	Dec	0.576
Interleukin-6 receptor subunit beta	0.1831	Dec	0.568
Keratin, type I cytoskeletal 19 (aa311-367)	0.1982	Inc	0.566
Kit ligand	0.7703	Inc	0.52
Lutropin subunit beta	0.9094	Dec	0.506
Lymphotactin	0.701	Inc	0.52
Macrophage colony-stimulating factor 1	0.6923	Inc	0.52
Matrilysin	0.5232	Dec	0.533
Metalloproteinase inhibitor 4	0.0288	Inc	0.613
Mix of Growth-regulated alpha, beta, and gamma proteins	0.6613	Inc	0.53
Mucin-16	0.7598	Inc	0.516
Neprilysin	0.2853	Inc	0.554
Neuronal cell adhesion molecule	0.3556	Inc	0.547
NF-kappa-B inhibitor alpha	0.4477	Inc	0.539
Osteocalcin	0.421	Inc	0.541
Oxidized low-density lipoprotein receptor 1	0.6712	Inc	0.529
Parathyroid hormone	0.9811	Inc	0.501
Platelet endothelial cell adhesion molecule	0.6301	Inc	0.524
Probetacellulin	0.4352	Inc	0.54
Pro-interleukin-16	0.9076	Dec	0.508
Prolactin	0.2906	Dec	0.555
Proprotein convertase subtilisin/kexin type 9	0.8167	Inc	0.512
Prostate-specific antigen	0.0493	Inc	0.6
Protein NOV homolog	0.8168	Dec	0.512
Protransforming growth factor alpha	0.9379	Dec	0.505
Serum amyloid P-component	0.0606	Dec	0.596
Somatotropin	0.0857	Inc	0.587
Stromal cell-derived factor 1	0.1301	Dec	0.602
Thymic stromal lymphopoietin	0.2869	Dec	0.572
Thyrotropin subunit beta	0.1617	Dec	0.571
Transmembrane glycoprotein NMB	0.4726	Dec	0.536
Tumor necrosis factor receptor superfamily member 8	0.1409	Dec	0.575
Vascular endothelial growth factor D	0.4156	Dec	0.541
Vascular endothelial growth factor receptor 1	0.0243	Dec	0.613
Vascular endothelial growth factor receptor 2	0.033	Dec	0.607

Vascular endothelial growth factor receptor 3	0.9207	Dec	0.505
WAP four-disulfide core domain protein 2	0.122	Inc	0.579

[0092] Table 4: Ischemic stroke (Cohort 1) vs. Mimics (Cohort 2)

Agouti-related protein	0.0625	Inc	0.525
Alpha-2 macroglobulin	0.0551	Dec	0.684
Alpha-fetoprotein	0.0635	Inc	0.513
Amphiregulin	0.0613	Inc	0.514
Angiopoietin-1 receptor	0.0611	Inc	0.518
Angiopoietin-related protein 3	0.0631	Dec	0.501
Angiopoietin-related protein 4	0.0615	Inc	0.575
Angiopoietin-related protein 6	0.0587	Dec	0.64
Bone morphogenetic protein 7	0.0612	Inc	0.509
Cadherin-3	0.0601	Dec	0.564
Cancer Antigen 15-3	0.0576	Dec	0.666
Cancer Antigen 19-9	0.0621	Dec	0.572
Carcinoembryonic antigen-related cell adhesion molecule 5	0.0634	Inc	0.521
C-C motif chemokine 1	0.0743	Inc	0.578
C-C motif chemokine 13	0.0518	Dec	0.73
C-C motif chemokine 15	0.0746	Dec	0.566
C-C motif chemokine 17	0.055	Dec	0.685
C-C motif chemokine 19	0.0599	Dec	0.609
C-C motif chemokine 26	0.0749	Dec	0.548
C-C motif chemokine 8	0.075	Dec	0.505
Choriogonadotropin subunit beta	0.057	Inc	0.676
Clusterin	0.0611	Inc	0.515
C-X-C motif chemokine 11	0.0629	Inc	0.503
C-X-C motif chemokine 6	0.0626	Inc	0.523
C-X-C motif chemokine 9	0.0623	Dec	0.537
Cyclin-dependent kinase inhibitor 1	0.0549	Inc	0.679
Endoglin	0.0613	Dec	0.5
Epiregulin	0.0609	Dec	0.541
Epithelial cell adhesion molecule	0.0608	Dec	0.536
Erythropoietin	0.061	Inc	0.521
Fatty acid-binding protein, liver	0.0628	Dec	0.525
Fibroblast growth factor 19	0.063	Inc	0.512
Fibroblast growth factor 21	0.0629	Dec	0.516
Fibroblast growth factor 23	0.0616	Dec	0.57

Follistatin	0.0522	Inc	0.718
Follitropin subunit beta	0.0613	Dec	0.573
Galectin-3	0.0612	Dec	0.504
Glial cell line-derived neurotrophic factor	0.0753	Dec	0.541
Heat shock protein beta-1	0.0584	Inc	0.613
Heparin-binding EGF-like growth factor	0.0611	Inc	0.532
Heparin-binding growth factor 1	0.0609	Inc	0.53
Hepatitis A virus cellular receptor 1	0.0612	Dec	0.509
Hepatocyte growth factor receptor	0.0602	Dec	0.559
Insulin receptor substrate 1	0.0569	Inc	0.645
Insulin-like growth factor-binding protein 1	0.0611	Inc	0.516
Insulin-like growth factor-binding protein 2	0.0579	Inc	0.624
Insulin-like growth factor-binding protein 4	0.0609	Inc	0.527
Intercellular adhesion molecule 3	0.0583	Dec	0.651
Interleukin-1 receptor type I	0.0563	Dec	0.675
Interleukin-1 receptor type II	0.0606	Dec	0.594
Interleukin-11	0.0617	Inc	0.562
Interleukin-21	0.075	Dec	0.54
Interleukin-28A	0.0735	Dec	0.609
Interleukin-29	0.0629	Inc	0.501
Interleukin-33	0.0751	Dec	0.519
Interleukin-4 receptor alpha chain	0.0584	Inc	0.641
Interleukin-6 receptor subunit alpha	0.0587	Dec	0.635
Interleukin-6 receptor subunit beta	0.057	Dec	0.665
Keratin, type I cytoskeletal 19 (aa311-367)	0.0612	Inc	0.596
Kit ligand	0.0749	Dec	0.541
Lutropin subunit beta	0.0609	Dec	0.586
Lymphotactin	0.0614	Inc	0.569
Macrophage colony-stimulating factor 1	0.0628	Dec	0.51
Matrilysin	0.0613	Dec	0.501
Metalloproteinase inhibitor 4	0.061	Inc	0.59
Mix of Growth-regulated alpha, beta, and gamma proteins	0.0745	Inc	0.538
Mucin-16	0.063	Inc	0.54
Neprilysin	0.06	Inc	0.567
Neuronal cell adhesion molecule	0.061	Dec	0.524
NF-kappa-B inhibitor alpha	0.0596	Inc	0.582
Osteocalcin	0.0626	Dec	0.523
Oxidized low-density lipoprotein receptor 1	0.0746	Inc	0.511
Parathyroid hormone	0.0625	Dec	0.526

Platelet endothelial cell adhesion molecule	0.0596	Inc	0.582
Probetacellulin	0.0614	Dec	0.512
Pro-interleukin-16	0.0749	Dec	0.547
Prolactin	0.0597	Dec	0.63
Proprotein convertase subtilisin/kexin type 9	0.061	Dec	0.52
Prostate-specific antigen	0.06	Inc	0.623
Protein NOV homolog	0.0613	Inc	0.502
Protransforming growth factor alpha	0.0606	Dec	0.555
Serum amyloid P-component	0.0564	Dec	0.662
Somatotropin	0.0604	Inc	0.597
Stromal cell-derived factor 1	0.0723	Dec	0.638
Thymic stromal lymphopoietin	0.0748	Dec	0.551
Thyrotropin subunit beta	0.0613	Dec	0.574
Transmembrane glycoprotein NMB	0.0594	Dec	0.586
Tumor necrosis factor receptor superfamily member 8	0.0616	Dec	0.563
Vascular endothelial growth factor D	0.0591	Dec	0.594
Vascular endothelial growth factor receptor 1	0.0573	Dec	0.66
Vascular endothelial growth factor receptor 2	0.0547	Dec	0.698
Vascular endothelial growth factor receptor 3	0.0616	Dec	0.563
WAP four-disulfide core domain protein 2	0.0587	Inc	0.649

[0093] Table 5: Ischemic stroke (Cohort 1) vs. Hemorrhagic stroke (Cohort 2)

Agouti-related protein	0.1385	Dec	0.594
Alpha-2 macroglobulin	0.7985	Dec	0.523
Alpha-fetoprotein	0.1983	Inc	0.583
Amphiregulin	0.6178	Dec	0.533
Angiopoietin-1 receptor	0.0202	Inc	0.644
Angiopoietin-related protein 3	0.0095	Inc	0.661
Angiopoietin-related protein 4	0.0515	Inc	0.623
Angiopoietin-related protein 6	0.0002	Inc	0.721
Bone morphogenetic protein 7	0.1847	Inc	0.584
Cadherin-3	0.4399	Inc	0.549
Cancer Antigen 15-3	0.0162	Inc	0.65
Cancer Antigen 19-9	0.1691	Inc	0.588
Carcinoembryonic antigen-related cell adhesion molecule 5	0.2414	Dec	0.575
C-C motif chemokine 1	0.4566	Inc	0.636
C-C motif chemokine 13	0.2668	Dec	0.698
C-C motif chemokine 15	0.043	Dec	0.815

C-C motif chemokine 17	0.6132	Dec	0.593
C-C motif chemokine 19	0.0043	Inc	0.671
C-C motif chemokine 26	0.4357	Dec	0.642
C-C motif chemokine 8	0.7102	Dec	0.568
Choriogonadotropin subunit beta	0.694	Inc	0.525
Clusterin	0.1715	Dec	0.587
C-X-C motif chemokine 11	0.0532	Dec	0.62
C-X-C motif chemokine 6	0.0846	Dec	0.607
C-X-C motif chemokine 9	0.0242	Inc	0.638
Cyclin-dependent kinase inhibitor 1	0.0101	Dec	0.659
Endoglin	0.1722	Inc	0.586
Epiregulin	0.2576	Dec	0.573
Epithelial cell adhesion molecule	0.2803	Inc	0.569
Erythropoietin	0.0001	Inc	0.724
Fatty acid-binding protein, liver	0.4918	Inc	0.544
Fibroblast growth factor 19	0.0412	Dec	0.629
Fibroblast growth factor 21	0.067	Inc	0.616
Fibroblast growth factor 23	<0.0001	Inc	0.735
Follistatin	0.1073	Dec	0.599
Follitropin subunit beta	0.2194	Inc	0.578
Galectin-3	0.7541	Inc	0.52
Glial cell line-derived neurotrophic factor	0.3	Dec	0.686
Heat shock protein beta-1	0.5681	Dec	0.537
Heparin-binding EGF-like growth factor	1	Dec	0.5
Heparin-binding growth factor 1	0.521	Inc	0.54
Hepatitis A virus cellular receptor 1	0.95	Inc	0.504
Hepatocyte growth factor receptor	0.3939	Inc	0.555
Insulin receptor substrate 1	0.0293	Dec	0.637
Insulin-like growth factor-binding protein 1	0.612	Inc	0.532
Insulin-like growth factor-binding protein 2	0.2352	Dec	0.575
Insulin-like growth factor-binding protein 4	0.0008	Dec	0.698
Intercellular adhesion molecule 3	0.671	Dec	0.54
Interleukin-1 receptor type I	0.1784	Inc	0.586
Interleukin-1 receptor type II	0.2245	Dec	0.578
Interleukin-11	0.4059	Dec	0.552
Interleukin-21	0.043	Dec	0.815
Interleukin-28A	0.5897	Dec	0.599
Interleukin-29	0.7651	Inc	0.519
Interleukin-33	0.5439	Dec	0.611

Interleukin-4 receptor alpha chain	0.4688	Inc	0.547
Interleukin-6 receptor subunit alpha	0.0487	Inc	0.624
Interleukin-6 receptor subunit beta	0.0513	Inc	0.623
Keratin, type I cytoskeletal 19 (aa311-367)	0.8219	Inc	0.515
Kit ligand	0.8646	Dec	0.531
Lutropin subunit beta	0.0287	Inc	0.636
Lymphotactin	0.3335	Dec	0.561
Macrophage colony-stimulating factor 1	0.5075	Dec	0.542
Matrilysin	0.0336	Dec	0.63
Metalloproteinase inhibitor 4	0.3346	Dec	0.591
Mix of Growth-regulated alpha, beta, and gamma proteins	0.2838	Dec	0.691
Mucin-16	0.0441	Inc	0.627
Neprilysin	0.0868	Inc	0.608
Neuronal cell adhesion molecule	0.0001	Inc	0.724
NF-kappa-B inhibitor alpha	0.7395	Inc	0.521
Osteocalcin	0.021	Inc	0.643
Oxidized low-density lipoprotein receptor 1	0.661	Dec	0.58
Parathyroid hormone	0.2408	Inc	0.574
Platelet endothelial cell adhesion molecule	0.1419	Inc	0.591
Probetacellulin	0.4909	Dec	0.545
Pro-interleukin-16	0.2503	Dec	0.704
Prolactin	0.0145	Inc	0.652
Proprotein convertase subtilisin/kexin type 9	0.3576	Dec	0.557
Prostate-specific antigen	0.4945	Inc	0.544
Protein NOV homolog	0.2976	Inc	0.566
Protransforming growth factor alpha	0.3192	Dec	0.679
Serum amyloid P-component	0.8767	Dec	0.514
Somatotropin	0.95	Dec	0.504
Stromal cell-derived factor 1	0.2187	Dec	0.716
Thymic stromal lymphopoietin	0.3952	Dec	0.654
Thyrotropin subunit beta	0.0185	Inc	0.645
Transmembrane glycoprotein NMB	0.0016	Inc	0.688
Tumor necrosis factor receptor superfamily member 8	0.1871	Dec	0.584
Vascular endothelial growth factor D	0.0559	Inc	0.617
Vascular endothelial growth factor receptor 1	0.8211	Inc	0.515
Vascular endothelial growth factor receptor 2	0.3077	Inc	0.566
Vascular endothelial growth factor receptor 3	0.193	Dec	0.583
WAP four-disulfide core domain protein 2	0.7918	Dec	0.517

[0094] Example 3. Use of analyte panels for assessing patients for stroke and/or TIA

[0095] Patients from the emergency department (ED) were enrolled in the study based on inclusion criteria consisting of: age > to 18 years, no recent history of trauma, having brain imaging performed, and experiencing a new neurologic symptom within 24 hours of blood draw. Exclusion criteria included hemoglobin < 12.5 g/dL for females and 13.5 g/dL for males, untreated systolic blood pressure < 90 mm Hg, and untreated diastolic blood pressure < 50 mm Hg. Stroke was defined as a persistent neurological deficit in a logical vascular distribution lasting 1.) ≥ 24 hours or 2.) < 24 hours but was either CT and/or MRI positive. Patients with a proven non-vascular condition and negative radiography interpreted by radiologists blinded to biomarker outcomes were stroke mimics. Patients with neurologic findings lasting < 24 hours in a logical vascular distribution, negative radiography, and negative workup for mimics were TIAs. Finally, those with intraparenchymal blood by radiograph were ICHs.

[0096] Plasma samples from each patient were collected at enrollment. The concentrations of the analyte in these samples were measured by standard immunoassay methods using commercially available assay reagents.

[0097] Statistical analysis was performed using SAS 9.3(SAS, Cary, NC). Descriptive statistics were obtained for demographic and clinical variables in each pathologic group: ischemic stroke, intracranial hemorrhage (ICH), TIA, and mimic. Means were compared across groups by one-way analysis of variance(ANOVA) tests if the variable was continuous and by χ^2 tests if categorical.

[0098] Univariate logistic regressions were performed with each biomarker to predict the likelihood of stroke versus mimic, stroke versus TIA + mimic, stroke + TIA versus mimic, stroke versus ICH, and stroke + ICH versus mimic. Biomarkers significant in univariate logistic regression in each group with a p-value ≤ 0.2 were retained and used to build multivariate logistic regression models within each comparison group. A stepwise selection method was employed to build the most parsimonious multivariate models maximizing predictive power and minimizing the number of covariates. Variables were retained if the F statistic was significant at p=0.05. The final variables were tested for multicollinearity by testing linear correlation(r>0.8) and variance inflation factor(>2.5). The area under the curve (AUC) of the receiver operating curve (ROC) of the models,

captured by the concordance index or c-statistic, was used as a measure of the overall discriminative capacity of each model.

[0099] The stroke vs. mimic model was set as a base model since the classification of both stroke and mimic patients was most rigorous, while the diagnosis of TIA was one of exclusion. Biomarker levels from a healthy cohort of subjects were a reference to test the discriminative capacity of the 3 variables to distinguish between patients with ischemic stroke and healthy patients. To internally validate this model, a SAS macro was adapted to bootstrap the parameter estimates and the c-statistic of the base model comparing ischemic stroke and mimics with 50 balanced re-samples generated.

[00100] In order to create one overall model, biomarkers significant in the multivariate models of ischemic stroke+TIA vs. mimic, ischemic stroke vs.TIA + mimic, ischemic stroke vs. ICH, and ischemic stroke + ICH vs. mimic were added to the base model incrementally and changes in bootstrapped c-statistics were monitored in the ischemic stroke vs. mimic comparison. Biomarkers contributing at least 1 percent increase in the c-statistic were retained. The overall model was used to generate boostrapped parameter estimates and c-statistics for the following comparisons: ischemic stroke versus mimic, ischemic stroke versus ICH, and ischemic stroke+ICH versus mimic. Sensitivities, specificities, positive predictive values(PPV), and negative predictive values(NPV) were determined for the probability of each outcome at which sensitivity is maximized with specificity at least 50%. These statistics were calculated at 30%, the approximate prevalence of stroke among patients who present to emergency rooms, and at the probability of the outcome in this study sample.[30]

[00101] To test the robustness of this model in face of the diagnostic uncertainty of the TIA group, the bootstrapped parameter estimates and intercept of the stroke vs. mimic comparison were used to predict TIA+stroke vs. mimic and stroke vs. TIA + mimic. Also, the model capacity to discriminate between anterior versus posterior and lacunar circulation ischemic strokes was also tested.

[00102] The predicted probabilities from the selected models were divided into deciles and the average predicted probability was plotted against the observed probability of patients within that decile. The distribution of predicted values yielded from inputting biomarker values of each patient into the model was plotted as a function of pathology.

[00103] Finally, the biomarkers of the base model were tested for collinearity(Pearson correlation > 0.4) with demographic variables known at presentation including age, gender, and race by. These covariates were used to build multivariate models which were compared to the final model. Additionally, a boxplot of each of the 5 final biomarkers was created displaying the mean, median, and interquartile ranges of each biomarker as a function of pathology.

[00104] Table 6 depicts the demographic and health status characteristics of each pathology group. In this sample, the mean age was 64.4 ±15.2 years (range 26 to 92); the average age among patients within each pathologic groups was not significantly different at α=0.05(p=.0772). The percent of patients in the aggregate sample who were male and female were 50.3% (N=84) and 49.7% (N=83), respectively. The proportion of each gender was not significantly different by pathologic group(p=.1416). Overall, there were 100 white patients (58.9%), 63 black patients (37.7%), and 4 of other race (2.4%). The proportion of patients in each pathologic group who were white in comparison to those who were black or other was significantly different by pathologic group (p=.0153). Among those with ICH, blacks and people of other races were overrepresented.

[00105] In terms of co-morbidities, nearly 80% of patients had hypertension uniformly across all groups, 30% had diabetes mellitus, and about 30% had known hyperlipidemia. A history of prior stroke was not significantly different in any of the groups of patients. Only the proportion of patients with atrial fibrillation was higher in both the ischemic stroke and TIA groups (p=.0263).

[00106] Table 6. Demographic and health status characteristics

Characteristic	Ischemic Stroke N=63	ICH N=26	TIA N=41	Mimic N=37	Healthy Patients N=70	P-value
Age (mean, SD)	67.1(15.3)	63.7(14.0)	63.05(15.9)	61.8(15.0)	44(16.0)	0.0772
Males	35(55.6)	16(61.5)	20(48.8)	13(35.1)	42(59.2)	0.1416
Race			0.0153			
White	40(63.5)	9(34.6)	30(73.2)	21(56.8)	49(69.0)	
Black + Other	23(36.5)	17(65.4)	11(26.8)	16(43.2)	21(31.0)	
Comorbidities						
Hypertension	52(82.5)	21(80.8)	30(73.2)	30(81.1)	-	0.6932
Diabetes mellitus	18(28.6)	7(26.9)	14(34.2)	14(37.8)	-	0.7256
Hyperlipidemia	17(27.0)	7(26.9)	12(29.3)	12(32.4)	-	0.9426
Atrial fibrillation	12(19.1)	0(0.0)	8(19.5)	2(5.4)	-	0.0263
Prior CVA/TIA	17(27.0)	11(42.3)	13(31.7)	12(32.4)	-	0.5711
Smoking	17(27.0)	2(7.7)	9(22.0)	7(18.9)	-	0.2347
Crack/Cocaine	3(4.8)	3(11.5)	1(2.4)	0(0.0)	-	0.1400

[00107] Table 2 contains the most common symptoms at presentation which included new onset of motor weakness (64.07%), speech/language problems (53.9%), sensory numbness (36.53%), headache (28.74%), altered mental status (22.16%), and visual disturbances (19.16%). A greater proportion of patients with ischemic strokes and TIAs were affected by speech/language difficulty (p<.0001). Patients with ICH were less likely to suffer from motor deficits thn patients of other diagnoses, particularly ischemic stroke(p=.0093). Sensory deficits were experienced less by ICH patients than by patients in the other groups(p=.0126). Patients with TIAs were less likely to have a change in mental status than those with the other diagnoses(p<.001 The overall average NIH stroke scale score was 5.1± 6.4. Patients diagnosed with ischemic stroke and ICH scored higher than TIAs and mimics (p<.0001). The average GCS was 14.2 ± 2.1, and was particularly low among ICH and ischemic stroke patients (p=.0018).

[00108] The median time from symptom onset to or last known usual state of health to serum biomarker blood draw was 10.25 hours (5, 18.75 interquartile range). Nearly 98% of patients received brain imaging which included CT, and 2% received MRI only. Among patients who had ischemic stroke, mimics, or TIA, 74 patients (44.1%) had a CT and an MRI. Among patients who had intracranial hemorrhage, 100% of patients had a CT.

[00109] The ABCD2 assessment and TOAST classification results are presented in Table 7 for patients diagnosed with TIAs and ischemic strokes, respectively. The

ABCD2 score predicts the risk of a stroke within 90 days after the TIA based on multiple covariates.[31]The average ABCD2 score for this TIA sample was 4.39 ± 1.16. The TOAST classification system is based on clinical symptoms and results from further investigation to determine the etiology of a stroke.[32] The majority of stroke events were due to an undetermined cause (41.3%) and secondary to atherosclerosis (33.3%), while 7% were due to a cardioembolic event. Among patients with ischemic strokes, nearly 57% occur in the anterior circulation, 11% comprise a lacunar infarct, and about 14% occur in the posterior circulation.

[00110] Table 7. Clinical and radiographic characteristics

Characteristic	Ischemic Stroke N=63	ICH N=26	TIA N=41	Mimic N=37	P-value
Symptoms					
Speech/Language	44(69.8)	5(19.2)	25(61.0)	16(43.2)	<.0001
Motor deficits	50(79.4)	12(46.2)	23(56.1)	22(59.5)	0.0093
Sensory deficits	23(36.5)	3(11.5)	21(51.2)	14(37.8)	0.0126
Visual deficits	12(19.1)	5(19.2)	6(14.6)	9(24.3)	0.7578
AMS	18(28.6)	13(50.0)	1(2.4)	5(13.5)	<.0001
Headache	12(19.1)	10(38.5)	13(31.7)	13(35.1)	0.1715
NIHSS at enrollment	6.75(6.5)	11.04(8.4)	1.46(2.5)	2.06(2.5)	<.0001
GCS (initial)	13.9(2.2)	12.54(3.2)	15(0.0)	14.81(0.6)	0.0018
TPA administered	21(33.0)	-	2(4.9)	2(5.4)	<.0001
ABCD2 score	-	-	4.39(1.2)	-	
TOAST criteria					
1(Atherosclerosis)	21(33.3)	-	-	-	
2(Cardioembolic)	7(11.1)	-	-	-	
3(small vessel occlusion)	2(3.2)	-	-	-	
4 (other determined cause)	7(11.1)	-	-	-	
5 (undetermined)	26(41.3)	-	-	-	
Vascular distribution					
Anterior	36(57.1)	-	-	-	
Lacunar	7(11.1)	-	-	-	
Posterior	9(14.3)	-	-	-	
>1 distribution	2(3.2)	-	-	-	

[00111] Discharge status, a proxy of functional status, varied greatly by diagnosis as is seen in Table 8. Among ischemic stroke and ICH patients, a smaller proportion were discharged home than among those with TIA and mimics (<.0001). The length of stay varied significantly by diagnosis, as patients with ischemic strokes and ICH remained inpatient longer. (p<.0001).

[00112] Table 8. Discharge destination as a proxy of functional status

Characteristic	Ischemic Stroke N=63	ICH N=26	TIA N=41	Mimic N=37	Normal N=70	P-value
Length of stay	6.47(5.6)	10.4(6.5)	2.4(2.9)	1.81(2.1)		<.0001
Discharge status						<.0001+
Home	34(53.97)	9(34.62)	37(90.25)	36 (97.29)		
Rehabilitation	11(17.46)	6(23.08)	2(4.88)	-		
SNF	8(12.7)	7(26.92)	2(4.88)	1(2.7)		
Hospice	5(7.94)	1(3.85)	-			
Deceased	5(7.94)	3(11.54)	-			

[00113] Multivariate logistic regression models built by stepwise selection from the pool of variables significant in the univariate analysis for each comparison group are displayed in Table 4. The log-odds of ischemic stroke versus mimic was predicted well by three biomarkers- eotaxin(p=0.0005), EGFR v1 (p=.0153), and S100A12(p=0.0206)- with a concordance index of 0.899. This model was used as the base model which was then bootstrapped for internal validation to obtain minimally biased parameter estimates and concordance statistics. The bootstrapped mean parameter estimates of the 3 aforementioned biomarkers comparing ischemic stroke versus mimic were the following: eotaxin(β=0.0023, p=0.0016), EGFR v1(β=0.0015, p=0.1107), and S100A12(β=-0.0726, p=0.1174). The bootstrapped mean concordance index was 0.8652.

[00114] The parameter estimates from this model were applied to predict the log-odds of being an ischemic stroke patient versus a healthy volunteer. When the coefficients were fixed to those of the ischemic stroke versus mimic model, the concordance index was 0.5 with a lack of fit (p<0.0001). However, when the coefficient were not fixed, these 3 variables predicted the log-odds of being an ischemic stroke patient versus healthy patient with a c-statististic of 0.842(Hosmer-Lemeshow test p=0.8908).

[00115] To create one overall model to predict TIA and ICHs as well, each variable significant by multivariate regression in the TIA and ICH analyses was added to the 3-variable model and the new overall model was bootstrapped each time in the ischemic stroke vs. mimic analysis. Only variables which individually contributed to an overall increase of the c-index by 1% were retained. Of the 4 variables significant in the TIA analyses, only metalloproteinase inhibitor-4(TIMP-4) met this criteria by increasing the c-

index by 5% upto 0.9019. Of the 6 variables significant in the ICH analyses, only prolactin met the standard by increasing the overall concordance index of the now 4-variable model to 0.9183(1.8% increase).

[00116] Table 9. Results of Multivariate Logistic Regressions

Biomarker	**AUC**	**P**
Angiopoietin 2	0.681	0.0092
EGFR	0.619	0.0117
Interleukin-1 receptor type II	0.581	0.0364
C-C motif chemokine 13	0.740	0.002
Overall model c-statistic	0.869	

Ischemic Stroke vs. TIA+ Mimic

Biomarker	**AUC**	**P**
Angiopoietin 2	0.680	0.0096
Eotaxin	0.728	0.0002
Interleukin-1 receptor type II	0.570	0.0139
Metalloproteinase inhibitor-4	0.608	0.0061
Overall model c-statistic	0.845	

Ischemic Stroke vs. Mimic

Biomarker	**AUC**	**P**
Eotaxin	0.809	0.0005
EGFR	0.696	0.0153
Protein S100 A12	0.607	0.0206
Overall model c-statistic	0.899	

Ischemic Stroke +ICH vs. Mimic

Biomarker	**AUC**	**P**
Growth differentiation factor-15	0.489	0.0092
Interleukin-1 receptor-like 1	0.718	0.0117
Interleukin-8	0.750	0.0364
Platelet endothelial cell adhesion molecule	0.538	0.002
Prolactin	0.75	0.0074
Overall model c-statistic	0.900	

Ischemic Stroke vs. ICH

Biomarker	AUC	P
Angiopoietin related protein 6	0.680	.0096
Interleukin-1 receptor-like 1	0.728	.0002
Overall model c-statistic	0.954	

[00117] Table 10. Results of Multivariate Logistic Regressions comparing Ischemic Stroke +ICH vs. Mimic and Ischemic Stroke vs. ICH.

Biomarker	AUC	P
Growth differentiation factor-15	0.489	0.0092
Interleukin-1 receptor-like 1	0.718	0.0117
Interleukin-8	0.750	0.0364
Platelet endothelial cell adhesion molecule	0.538	0.002
Prolactin	0.75	0.0074
Overall model c-statistic	0.900	

[00118] The final model consisted of 5 variables: eotaxin, EGFR, S100A12, TIMP-4, and prolactin. This model was internally validated with the bootstrapping technique in three comparison groups.(Table 11) When discriminating between ischemic strokes and mimics, the 5-variable model yielded a bootstrapped c-statistic of 0.9183(95%CI: 0.909, 0.9276); a goodness of fit confirmed with a p of 0.1416 (Hosmer-Lemeshow test); an overall model likelihood ratio χ^2 of 49.7161($p<0.0001$). When comparing the log-odds of ischemic stroke + ICH versus mimic, the 5-variable model bootstrapped c-statistic was 0.9273(95% CI: 0.919, 0.9355); goodness of fit was confirmed with a Hosmer-Lemeshow test p of 0.5802; overall model likelihood ratio χ^2 was 55.8567($p<.0001$). Finally, when the 5 variables were used to regress the log-odds of ICH versus ischemic stroke, the bootstrapped c-statistic was 0.8958(95%CI: 0.8806, 0.911); its goodness of fit confirmed by a Hosmer-Lemeshow test p-value of 0.9935; an overall model likelihood ratio χ^2 of 13.5990 ($p=0.0184$).

[00119] Table 11. Results of Internal Validation by Bootstrapping

Stroke v. Mimic

Biomarker	**Parameter estimate (95% CI)**	**P**
Eotaxin	.003 (-.00141, .005)	0.0021
EGFR v1	.0018 (-.0013, .004)	0.1914
TIMP-4	-.0004 (-.0008, .0005)	0.3325
Prolactin	.0001 (-.0001, .0002)	0.2325
S100A12	-.1253 (-.2225, .1241)	0.0621
Intercept	-4.1563 (-7.2863, 1.2487)	0.0503
C-statistic	0.9183(0.909, 0.9276)	

Stroke + ICH v. Mimic

Biomarker	**Parameter estimate (95% CI)**	**P**
Eotaxin	.0032(-.0013, .0145)	0.0012
EGFR v1	.0019(-.0013, .004)	0.1877
TIMP-4	-.0004(-.0008, .0004)	0.3167
Prolactin	.0001(-.0002, .0002)	0.2158
S100A12	-.1278(-.2268, .1249)	0.0607
Intercept	-4.31(-7.334,1.0617)	0.0351
C-statistic	0.9273(0.919, 0.9355)	

Stroke v. ICH

Biomarker	**Parameter estimate (95% CI)**	**P**
Eotaxin	.00658(-.0083, .0145)	0.26764
EGFR v1	-.00089(-.0148, .0176)	0.45978
TIMP-4	-.00056(-.0018, .0018)	0.31563
Prolactin	.0007(-.0038, .0035)	0.31035
S100A12	.0162(-.0918, .1074)	
Intercept	1.8919(-21.7902, 18.3112)	
C-statistic	0.8958(0.8806, 0.911)	

[00120] Table 12 contains the sensitivities, specificities, PPV, and NPV from these models. The robustness of the 5-variable model was tested by the addition of TIA to the ischemic stroke group and then to the mimic group while keeping fixed the parameter estimates from the 5-variable model. When TIA patients were grouped with ischemic

strokes, the c-statistic was 0.812, however, the model goodness of fit($p<0.0001$) and likelihood ratio χ^2(0.711, p=0.9824) were poor. When TIA patients were grouped with mimic patients, the model fared better with a c-statistic of 0.824, model goodness of fit confirmed at p=0.4980, and likelihood ratio of 45.44($p<0.0001$).

[00121] The 5-variable model also predicted the log-odds of an anterior circulation versus a posterior circulation or lacunar stroke with fair accuracy(c=0.733). Goodness of fit was established with a Hosmer and Lemeshow probability of 0.2895. The only covariate with a Wald χ^2 bordering statistical significance was prolactin(p=0.0807). Possible associations between each biomarker and age, race, and gender were tested with linear regressions. Prolactin was significantly associated with age (0.0362) with an adjusted R-squared of 0.0216; TIMP-4 with age ($p<.0001$, adjusted R-squared=0.2525); and TIMP-4 with gender ($p<0.0001$, adjusted R-squared 0.0882). None of the 5 markers were significantly collinear($r > 0.4$) with each other.

[00122] Table 12. Discriminative capacity of the 5-biomarker model and the model with 5 biomarkers plus age, race and sex

Probability	SENSITIVITY	SPECIFICITY	PPV	NPV
Ischemic Stroke vs. Mimic Predicted by Biomarkers				
0.10	90.6	50.0	51.8	90.0
0.30	81.3	77.8	68.4	87.5
0.38†	78.1	79.6	69.4	86.0
Ischemic Stroke vs. Mimic Predicted by Biomarkers, Age, Race, and Gender				
0.10	93.5	75.9	69.0	95.3
0.30	90.3	85.2	77.1	93.9
0.38†	87.1	85.2	77.1	92
Ischemic Stroke + ICH vs. Mimic Predicted by Biomarkers				
0.10	90.6	54.8	51.9	91.9
0.30	78.1	80.6	67.6	87.7
0.53†	71.9	88.7	76.7	85.9
Ischemic Stroke + ICH vs. Mimic Predicted by Biomarkers, Age, Race, and Gender				
0.10	93.5	79.0	69	96.1
0.30	90.3	87.1	77.8	94.7
0.53†	80.6	88.7	78.1	91.2

[00123] In this prospective, observational study, 5 biomarkers (eotaxin, epidermal growth factor receptor, S100A12, TIMP-4, and prolactin) robustly differentiated outcomes of interest including ischemic stroke, ICH, and stroke mimics. These are largely novel and not representative of the stroke biomarkers which dominate the literature such as markers of glial activation including S100B and GFAP and markers of neuronal injury such as NSE. The biomarkers in the final stroke model have pathophysiologic roles which lend biologic plausibility to their statistical association with stroke. Eotaxin is a potent chemokine for eosinophils and other inflammatory cells. Treatment of vascular endothelial cells with TNF-alpha resulted in a 20-fold induction of smooth muscles expression of eotaxin in human atheroma as well as an increase in macrophage and mast cell expression of CCR3, its receptor, suggesting eotaxin recruits inflammatory cells in atheromas. Another study demonstrated an increase in eotaxin in ischemic stroke patients versus healthy controls. Epidermal growth factor receptor(EGFR) and its ligands may play a role in regulation of genes associated with reactive gliosis. After an ischemic event, proliferating glial cells abundant in the infarcted brain and astrocytes in the periphery of the infarct were highly immunoreactive to EGFR.

[00124] As a chemoattractant for monocytic cells, S100A12 is an inflammatory response mediator. Its receptor, RAGE (receptor for advanced glycation end products), activates transcription factors that play an important role in protection from oxidative stress such as NF-κB. S100A12 was found to dramatically induce neurite outgrowth from rat embryonic hippocampal cells. Finally, plasma levels of S100A12 were higher in patients with carotid atherosclerosis and highest in patients with most recent symptoms.

[00125] TIMP-4 irreversibly inactivates metalloproteinases and is expressed in astrocytes, monocytes, platelets, smooth muscle cells, and endothelial cells. It is a major intraplatelet matrix metalloproteinase inhibitor, thus involved in regulating platelet recruitment and aggregation.

[00126] Prolactin is involved in platelet activation as P-selectin expression and platelet aggregation increase in its presence and in acute ischemic stroke patients.There is an isoform of the prolactin receptor on platelets, suggesting a mechanism similar to ADP-induced platelet aggregation.Moreover, patients with prolactinomas have an increased incidence of deep venous thromboembolism indicating that elevated prolactin may contribute to hypercoagulability.

[00127] One important feature of this biomarker test is its NPV. A clinically relevant question that an emergency medicine physician faces when presented with a patient with an acute neurologic deficit is whether the patient can be safely sent home. If the probability of stroke is set at its prevalence among patients who present to the emergency department with a neurologic problem, then the proportion of patients with a negative test who are correctly diagnosed by the biomarker test coupled with information about age, gender, and race is 94.7 percent when comparing ischemic stroke and ICH patients to mimics

[00128] While the invention has been described and exemplified in sufficient detail for those skilled in this art to make and use it, various alternatives, modifications, and improvements should be apparent without departing from the spirit and scope of the invention. The examples provided herein are representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Modifications therein and other uses will occur to those skilled in the art. These modifications are encompassed within the spirit of the invention and are defined by the scope of the claims.

[00129] It will be readily apparent to a person skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

[00130] All patents and publications mentioned in the specification are indicative of the levels of those of ordinary skill in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

[00131] The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the

scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

[00132] Other embodiments are set forth within the following claims.

We claim:

1. A method of diagnosing cerebral injury in a subject, or assigning a likelihood of a future outcome to a subject diagnosed with a cerebral injury, comprising:

performing one or more assays configured to detect one or more biomarkers selected from the group consisting of Agouti-related protein, Alpha-2 macroglobulin, Alpha-fetoprotein, Amphiregulin, Angiopoietin-1 receptor, Angiopoietin-related protein 3, Angiopoietin-related protein 4, Angiopoietin-related protein 6, Bone morphogenetic protein 7, Cadherin-3, Cancer Antigen 15-3, Cancer Antigen 19-9, Carcinoembryonic antigen-related cell adhesion molecule 5, C-C motif chemokine 1, C-C motif chemokine 13, C-C motif chemokine 15, C-C motif chemokine 17, C-C motif chemokine 19, C-C motif chemokine 26, C-C motif chemokine 8, Choriogonadotropin subunit beta, Clusterin, C-X-C motif chemokine 11, C-X-C motif chemokine 6, C-X-C motif chemokine 9, Cyclin-dependent kinase inhibitor 1, Endoglin, Epiregulin, Epithelial cell adhesion molecule, Erythropoietin, Fatty acid-binding protein, liver, Fibroblast growth factor 19, Fibroblast growth factor 21, Fibroblast growth factor 23, Follistatin, Follitropin subunit beta, Galectin-3, Glial cell line-derived neurotrophic factor, Heat shock protein beta-1, Heparin-binding EGF-like growth factor, Heparin-binding growth factor 1, Hepatitis A virus cellular receptor 1, Hepatocyte growth factor receptor, Insulin receptor substrate 1, Insulin-like growth factor-binding protein 1, Insulin-like growth factor-binding protein 2, Insulin-like growth factor-binding protein 4, Intercellular adhesion molecule 3, Interleukin-1 receptor type I, Interleukin-1 receptor type II, Interleukin-11, Interleukin-21, Interleukin-28A, Interleukin-29, Interleukin-33, Interleukin-4 receptor alpha chain, Interleukin-6 receptor subunit alpha, Interleukin-6 receptor subunit beta, Keratin, type I cytoskeletal 19 (aa311-367), Kit ligand, Lutropin subunit beta, Lymphotactin, Macrophage colony-stimulating factor 1, Matrilysin, Metalloproteinase inhibitor 4, Growth-regulated alpha, beta, and gamma proteins (total), Mucin-16, Neprilysin, Neuronal cell adhesion molecule, NF-kappa-B inhibitor alpha, Osteocalcin, Oxidized low-density lipoprotein receptor 1, Parathyroid hormone, Platelet endothelial cell adhesion molecule, Probetacellulin, Pro-interleukin-16, Prolactin, Proprotein convertase subtilisin/kexin type 9, Prostate-specific antigen, Protein NOV homolog, Protransforming growth factor alpha, Serum amyloid P-component, Somatotropin, Stromal cell-derived factor 1, Thymic stromal lymphopoietin, Thyrotropin subunit beta, Transmembrane glycoprotein NMB, Tumor necrosis factor receptor superfamily member

8, Vascular endothelial growth factor D, Vascular endothelial growth factor receptor 1, Vascular endothelial growth factor receptor 2, Vascular endothelial growth factor receptor 3, and WAP four-disulfide core domain protein 2 on a body fluid sample obtained from the subject to provide one or more assay result(s); and

correlating the assay result(s) to the occurrence or nonoccurrence of the cerebral injury in the subject or likelihood of the future outcome to the subject.

2. A method according to claim 1, wherein the performing step comprises introducing the body fluid sample obtained from the subject into an assay instrument which (i) contacts the body fluid sample with one or more binding reagents corresponding to the biomarker(s) being assayed, wherein each biomarker which is assayed binds to its respective specific binding reagent in an amount related to its concentration in the body fluid sample, (ii) generates one or more assay results indicative of binding of each biomarker which is assayed to its respective specific binding reagent; and (iii) displays the one or more assay results as a quantitative result in a human-readable form.

3. A method according to claim 1 or 2, wherein the future outcome is mortality.

4. A method according to claim 1 or 2, wherein the cerebral injury is selected from the group consisting of ischemic stroke, hemorrhagic stroke, TIA, or traumatic brain injury.

5. A method according to claim 1 or 2, wherein the correlating step comprises determining the concentration of each biomarker which is assayed, and individually comparing each biomarker concentration to a corresponding threshold level for that biomarker.

6. A method according to claim 5, wherein the assay instrument performs the correlating step, which comprises determining the concentration of each biomarker which is assayed, individually comparing each biomarker concentration to a corresponding threshold level for that biomarker, and displaying an indication of whether each biomarker does or does not exceed its corresponding threshold in a human-readable form.

7. A method according to claim 2, wherein a plurality of the biomarkers are measured, wherein the assay instrument performs the correlating step, which comprises determining the concentration of each of the plurality of biomarkers, calculating a single value based on the concentration of each of the plurality of biomarkers, comparing the single value to a corresponding threshold level and displaying an indication of whether the single value does or does not exceed its corresponding threshold in a human-readable form.

8. A method according to one of claims 1-7, wherein method provides a sensitivity or specificity of at least 0.7 for the identification of a cerebral injury selected from the group consisting of ischemic stroke, hemorrhagic stroke, TIA, or traumatic brain injury when compared to normal subjects.

9. A method according to one of claims 1-7, wherein method provides a sensitivity or specificity of at least 0.7 for the identification of a cerebral injury selected from the group consisting of ischemic stroke, hemorrhagic stroke, TIA, or traumatic brain injury when compared to subjects exhibiting symptoms that mimic stroke symptoms.

10. A method according to one of claims 1-7, wherein method provides a sensitivity or specificity of at least 0.7 for the identification of a cerebral injury selected from the group consisting of ischemic stroke and hemorrhagic stroke when compared to subjects exhibiting symptoms that mimic stroke symptoms and subjects suffering from TIA.

11. A method according to one of claims 1-7, wherein method provides a sensitivity or specificity of at least 0.7 for the identification of a hemorrhagic strokewhen compared to subjects exhibiting symptoms that mimic stroke symptoms and subjects suffering from TIA or ischemic stroke.

12. A method according one of claims 1-7, wherein method provides a sensitivity or specificity of at least 0.7 for the identification of a ischemic strokewhen compared to subjects exhibiting symptoms that mimic stroke symptoms and subjects suffering from TIA or hemorrhagic stroke.

13. A method according to one of claims 1-7, wherein method provides an odds ratio of at least 2 for the prognostic risk of mortality.

14. A method according to one of claims 1-13, wherein the sample is selected from the group consisting of blood, serum, and plasma.

15. A method according to one of claims 1-14, wherein the cerebral injury is ischemic stroke.

16. A method according to one of claims 1-14, wherein the cerebral injury is hemorrhagic stroke.

17. A method according to one of claims 1-14, wherein the cerebral injury is traumatic brain injury.

18. A method for evaluating biomarker levels in a body fluid sample, comprising:

obtaining a body fluid sample from a subject selected for evaluation based on a determination that the subject is experiencing symptoms indicative of a possible ischemic stroke, hemorrhagic stroke, TIA, or traumatic brain injury and is in need of evaluation to determine the cause of such symptoms; and

performing one or more analyte binding assays configured to detect one or more biomarkers selected from the group consisting of Agouti-related protein, Alpha-2 macroglobulin, Alpha-fetoprotein, Amphiregulin, Angiopoietin-1 receptor, Angiopoietin-related protein 3, Angiopoietin-related protein 4, Angiopoietin-related protein 6, Bone morphogenetic protein 7, Cadherin-3, Cancer Antigen 15-3, Cancer Antigen 19-9, Carcinoembryonic antigen-related cell adhesion molecule 5, C-C motif chemokine 1, C-C motif chemokine 13, C-C motif chemokine 15, C-C motif chemokine 17, C-C motif chemokine 19, C-C motif chemokine 26, C-C motif chemokine 8, Choriogonadotropin subunit beta, Clusterin, C-X-C motif chemokine 11, C-X-C motif chemokine 6, C-X-C motif chemokine 9, Cyclin-dependent kinase inhibitor 1, Endoglin, Epiregulin, Epithelial cell adhesion molecule, Erythropoietin, Fatty acid-binding protein, liver, Fibroblast growth factor 19, Fibroblast growth factor 21, Fibroblast growth factor 23, Follistatin, Follitropin subunit beta, Galectin-3, Glial cell line-derived neurotrophic factor, Heat shock protein beta-1, Heparin-binding EGF-like growth factor, Heparin-binding growth factor 1, Hepatitis A virus cellular receptor 1, Hepatocyte growth factor receptor, Insulin receptor substrate 1, Insulin-like growth factor-binding protein 1, Insulin-like growth factor-binding protein 2, Insulin-like growth factor-binding protein 4, Intercellular adhesion molecule 3, Interleukin-1 receptor type I, Interleukin-1 receptor type II,

Interleukin-11, Interleukin-21, Interleukin-28A, Interleukin-29, Interleukin-33, Interleukin-4 receptor alpha chain, Interleukin-6 receptor subunit alpha, Interleukin-6 receptor subunit beta, Keratin, type I cytoskeletal 19 (aa311-367), Kit ligand, Lutropin subunit beta, Lymphotactin, Macrophage colony-stimulating factor 1, Matrilysin, Metalloproteinase inhibitor 4, Growth-regulated alpha, beta, and gamma proteins (total), Mucin-16, Neprilysin, Neuronal cell adhesion molecule, NF-kappa-B inhibitor alpha, Osteocalcin, Oxidized low-density lipoprotein receptor 1, Parathyroid hormone, Platelet endothelial cell adhesion molecule, Probetacellulin, Pro-interleukin-16, Prolactin, Proprotein convertase subtilisin/kexin type 9, Prostate-specific antigen, Protein NOV homolog, Protransforming growth factor alpha, Serum amyloid P-component, Somatotropin, Stromal cell-derived factor 1, Thymic stromal lymphopoietin, Thyrotropin subunit beta, Transmembrane glycoprotein NMB, Tumor necrosis factor receptor superfamily member 8, Vascular endothelial growth factor D, Vascular endothelial growth factor receptor 1, Vascular endothelial growth factor receptor 2, Vascular endothelial growth factor receptor 3, and WAP four-disulfide core domain protein 2 by introducing the body fluid sample obtained from the subject into an assay instrument which (i) contacts the body fluid sample with one or more binding reagents corresponding to the biomarker(s) being assayed, wherein each biomarker which is assayed binds to its respective specific binding reagent in an amount related to its concentration in the body fluid sample, (ii) generates one or more assay results indicative of binding of each biomarker which is assayed to its respective specific binding reagent; and (iii) displays the one or more assay results as a quantitative result in a human-readable form.

19. A method according to claim 18, wherein the assay result(s) are displayed as a concentration of each biomarker which is assayed.

20. A method according to claim 19, wherein the assay instrument further individually compares each biomarker concentration to a corresponding threshold level for that biomarker, and displays an indication of whether each biomarker does or does not exceed its corresponding threshold in a human-readable form.

21. A method according to claim 18, wherein a plurality of the biomarkers are measured, and wherein the assay results(s) comprise a single value calculated using a function that converts the concentration of each of the plurality of biomarkers into a single value.

22. A method according to claim 21, wherein the assay instrument further compares the single value to a corresponding threshold level and displays an indication of whether the single value does or does not exceed its corresponding threshold in a human-readable form.

23. A method for evaluating biomarker levels in a body fluid sample, comprising:

obtaining a body fluid sample from a subject selected for evaluation based on a determination that the subject has been diagnosed with an ischemic stroke, hemorrhagic stroke, TIA, or traumatic brain injury and is in need of risk stratification to determine a risl of one or more outcomes due to the ischemic stroke, hemorrhagic stroke, TIA, or traumatic brain injury; and

performing one or more analyte binding assays configured to detect one or more biomarkers selected from the group consisting of Agouti-related protein, Alpha-2 macroglobulin, Alpha-fetoprotein, Amphiregulin, Angiopoietin-1 receptor, Angiopoietin-related protein 3, Angiopoietin-related protein 4, Angiopoietin-related protein 6, Bone morphogenetic protein 7, Cadherin-3, Cancer Antigen 15-3, Cancer Antigen 19-9, Carcinoembryonic antigen-related cell adhesion molecule 5, C-C motif chemokine 1, C-C motif chemokine 13, C-C motif chemokine 15, C-C motif chemokine 17, C-C motif chemokine 19, C-C motif chemokine 26, C-C motif chemokine 8, Choriogonadotropin subunit beta, Clusterin, C-X-C motif chemokine 11, C-X-C motif chemokine 6, C-X-C motif chemokine 9, Cyclin-dependent kinase inhibitor 1, Endoglin, Epiregulin, Epithelial cell adhesion molecule, Erythropoietin, Fatty acid-binding protein, liver, Fibroblast growth factor 19, Fibroblast growth factor 21, Fibroblast growth factor 23, Follistatin, Follitropin subunit beta, Galectin-3, Glial cell line-derived neurotrophic factor, Heat shock protein beta-1, Heparin-binding EGF-like growth factor, Heparin-binding growth factor 1, Hepatitis A virus cellular receptor 1, Hepatocyte growth factor receptor, Insulin receptor substrate 1, Insulin-like growth factor-binding protein 1, Insulin-like growth factor-binding protein 2, Insulin-like growth factor-binding protein 4, Intercellular adhesion molecule 3, Interleukin-1 receptor type I, Interleukin-1 receptor type II, Interleukin-11, Interleukin-21, Interleukin-28A, Interleukin-29, Interleukin-33, Interleukin-4 receptor alpha chain, Interleukin-6 receptor subunit alpha, Interleukin-6 receptor subunit beta, Keratin, type I cytoskeletal 19 (aa311-367), Kit ligand, Lutropin subunit beta, Lymphotactin, Macrophage colony-stimulating factor 1, Matrilysin, Metalloproteinase inhibitor 4, Growth-regulated alpha, beta, and gamma proteins (total),

Mucin-16, Neprilysin, Neuronal cell adhesion molecule, NF-kappa-B inhibitor alpha, Osteocalcin, Oxidized low-density lipoprotein receptor 1, Parathyroid hormone, Platelet endothelial cell adhesion molecule, Probetacellulin, Pro-interleukin-16, Prolactin, Proprotein convertase subtilisin/kexin type 9, Prostate-specific antigen, Protein NOV homolog, Protransforming growth factor alpha, Serum amyloid P-component, Somatotropin, Stromal cell-derived factor 1, Thymic stromal lymphopoietin, Thyrotropin subunit beta, Transmembrane glycoprotein NMB, Tumor necrosis factor receptor superfamily member 8, Vascular endothelial growth factor D, Vascular endothelial growth factor receptor 1, Vascular endothelial growth factor receptor 2, Vascular endothelial growth factor receptor 3, and WAP four-disulfide core domain protein 2 by introducing the body fluid sample obtained from the subject into an assay instrument which (i) contacts the body fluid sample with one or more binding reagents corresponding to the biomarker(s) being assayed, wherein each biomarker which is assayed binds to its respective specific binding reagent in an amount related to its concentration in the body fluid sample, (ii) generates one or more assay results indicative of binding of each biomarker which is assayed to its respective specific binding reagent; and (iii) displays the one or more assay results as a quantitative result in a human-readable form.

24. A method according to claim 23, wherein the assay result(s) are displayed as a concentration of each biomarker which is assayed.

25. A method according to claim 24, wherein the assay instrument further individually compares each biomarker concentration to a corresponding threshold level for that biomarker, and displays an indication of whether each biomarker does or does not exceed its corresponding threshold in a human-readable form.

26. A method according to claim 23, wherein a plurality of the biomarkers are measured, and wherein the assay results(s) comprise a single value calculated using a function that converts the concentration of each of the plurality of biomarkers into a single value.

27. A method according to claim 26, wherein the assay instrument further compares the single value to a corresponding threshold level and displays an indication of whether the single value does or does not exceed its corresponding threshold in a human-readable form.

28. A method according to one of claims 23-26, wherein the subject is selected for evaluation of a mortality risk within a period selected from the group consisting of 21 days, 14 days, 7 days, 5 days, 96 hours, 72 hours, 48 hours, 36 hours, 24 hours, and 12 hours.

29. A method according to one of claims 18-28, wherein the plurality of assays are immunoassays performed by (i) introducing the body fluid sample into an assay device comprising a plurality of antibodies, at least one of which binds to each biomarker which is assayed, and (ii) generating an assay result indicative of binding of each biomarker to its respective antibody.

30. A method according to one of claims 1-29, further comprising treating the patient for ischemic stroke, hemorrhagic stroke, TIA, or traumatic brain injury based on the assay result(s) obtained.

31. A system for evaluating biomarker levels, comprising:

a plurality of reagents which specifically bind for detection the plurality of biomarkers selected from the group consisting of Agouti-related protein, Alpha-2 macroglobulin, Alpha-fetoprotein, Amphiregulin, Angiopoietin-1 receptor, Angiopoietin-related protein 3, Angiopoietin-related protein 4, Angiopoietin-related protein 6, Bone morphogenetic protein 7, Cadherin-3, Cancer Antigen 15-3, Cancer Antigen 19-9, Carcinoembryonic antigen-related cell adhesion molecule 5, C-C motif chemokine 1, C-C motif chemokine 13, C-C motif chemokine 15, C-C motif chemokine 17, C-C motif chemokine 19, C-C motif chemokine 26, C-C motif chemokine 8, Choriogonadotropin subunit beta, Clusterin, C-X-C motif chemokine 11, C-X-C motif chemokine 6, C-X-C motif chemokine 9, Cyclin-dependent kinase inhibitor 1, Endoglin, Epiregulin, Epithelial cell adhesion molecule, Erythropoietin, Fatty acid-binding protein, liver, Fibroblast growth factor 19, Fibroblast growth factor 21, Fibroblast growth factor 23, Follistatin, Follitropin subunit beta, Galectin-3, Glial cell line-derived neurotrophic factor, Heat shock protein beta-1, Heparin-binding EGF-like growth factor, Heparin-binding growth factor 1, Hepatitis A virus cellular receptor 1, Hepatocyte growth factor receptor, Insulin receptor substrate 1, Insulin-like growth factor-binding protein 1, Insulin-like growth factor-binding protein 2, Insulin-like growth factor-binding protein 4, Intercellular adhesion molecule 3, Interleukin-1 receptor type I, Interleukin-1 receptor type II, Interleukin-11, Interleukin-21, Interleukin-28A, Interleukin-29, Interleukin-33, Interleukin-4 receptor

alpha chain, Interleukin-6 receptor subunit alpha, Interleukin-6 receptor subunit beta, Keratin, type I cytoskeletal 19 (aa311-367), Kit ligand, Lutropin subunit beta, Lymphotactin, Macrophage colony-stimulating factor 1, Matrilysin, Metalloproteinase inhibitor 4, Growth-regulated alpha, beta, and gamma proteins (total), Mucin-16, Neprilysin, Neuronal cell adhesion molecule, NF-kappa-B inhibitor alpha, Osteocalcin, Oxidized low-density lipoprotein receptor 1, Parathyroid hormone, Platelet endothelial cell adhesion molecule, Probetacellulin, Pro-interleukin-16, Prolactin, Proprotein convertase subtilisin/kexin type 9, Prostate-specific antigen, Protein NOV homolog, Protransforming growth factor alpha, Serum amyloid P-component, Somatotropin, Stromal cell-derived factor 1, Thymic stromal lymphopoietin, Thyrotropin subunit beta, Transmembrane glycoprotein NMB, Tumor necrosis factor receptor superfamily member 8, Vascular endothelial growth factor D, Vascular endothelial growth factor receptor 1, Vascular endothelial growth factor receptor 2, Vascular endothelial growth factor receptor 3, and WAP four-disulfide core domain protein 2;

an assay instrument configured to (i) receive a body fluid sample, (ii) contact the plurality of reagents with the body fluid sample and (iii) generate and quantitatively display in human readable form one or more assay results indicative of binding of each biomarker which is assayed to a respective specific binding reagent in the plurality of reagents.

32. A system according to claim 30 wherein the reagents comprise a plurality of antibodies, at least one of which binds to each of the biomarkers which are assayed.

33. A system according to claim 31 wherein assay instrument comprises an assay device and an assay device reader, wherein the plurality of antibodies are immobilized at a plurality of predetermined locations within the assay device, wherein the assay device is configured to receive the body fluid sample such that the body fluid sample contacts the plurality of predetermined locations, and wherein the assay device reader interrogates the plurality of predetermined locations to generate the assay results.

34. A method of diagnosing cerebral injury in a subject, or assigning a likelihood of a future outcome to a subject diagnosed with a cerebral injury, comprising:

performing one or more assays configured to detect one or more biomarkers selected from the group consisting of eotaxin, epidermal growth factor receptor, S100A12, TIMP-4, and prolactin on a body fluid sample obtained from the subject to provide one or more assay result(s); and

correlating the assay result(s) to the occurrence or nonoccurrence of the cerebral injury in the subject or likelihood of the future outcome to the subject.

35. A method according to claim 34, wherein the performing step comprises introducing the body fluid sample obtained from the subject into an assay instrument which (i) contacts the body fluid sample with one or more binding reagents corresponding to the biomarker(s) being assayed, wherein each biomarker which is assayed binds to its respective specific binding reagent in an amount related to its concentration in the body fluid sample, (ii) generates one or more assay results indicative of binding of each biomarker which is assayed to its respective specific binding reagent; and (iii) displays the one or more assay results as a quantitative result in a human-readable form.

36. A method according to claim 34 or 35, wherein the future outcome is mortality.

37. A method according to claim 34 or 35, wherein the cerebral injury is selected from the group consisting of ischemic stroke, hemorrhagic stroke, TIA, or traumatic brain injury.

38. A method according to claim 34 or 35, wherein the correlating step comprises determining the concentration of each biomarker which is assayed, and individually comparing each biomarker concentration to a corresponding threshold level for that biomarker.

39. A method according to claim 38, wherein the assay instrument performs the correlating step, which comprises determining the concentration of each biomarker which is assayed, individually comparing each biomarker concentration to a corresponding threshold level for that biomarker, and displaying an indication of whether each biomarker does or does not exceed its corresponding threshold in a human-readable form.

40. A method according to claim 35, wherein a plurality of the biomarkers are measured, wherein the assay instrument performs the correlating step, which comprises determining the concentration of each of the plurality of biomarkers, calculating a single value based on the concentration of each of the plurality of biomarkers, comparing the single value to a corresponding threshold level and displaying an indication of whether the single value does or does not exceed its corresponding threshold in a human-readable form.

41. A method according to one of claims 34-40, wherein method provides a sensitivity or specificity of at least 0.7 for the identification of a cerebral injury selected from the group consisting of ischemic stroke, hemorrhagic stroke, TIA, or traumatic brain injury when compared to normal subjects.

42. A method according to one of claims 34-40, wherein method provides a sensitivity or specificity of at least 0.7 for the identification of a cerebral injury selected from the group consisting of ischemic stroke, hemorrhagic stroke, TIA, or traumatic brain injury when compared to subjects exhibiting symptoms that mimic stroke symptoms.

43. A method according to one of claims 34-40, wherein method provides a sensitivity or specificity of at least 0.7 for the identification of a cerebral injury selected from the group consisting of ischemic stroke and hemorrhagic stroke when compared to subjects exhibiting symptoms that mimic stroke symptoms and subjects suffering from TIA.

44. A method according to one of claims 34-40, wherein method provides a sensitivity or specificity of at least 0.7 for the identification of a hemorrhagic strokewhen compared to subjects exhibiting symptoms that mimic stroke symptoms and subjects suffering from TIA or ischemic stroke.

45. A method according one of claims 34-40, wherein method provides a sensitivity or specificity of at least 0.7 for the identification of a ischemic strokewhen compared to subjects exhibiting symptoms that mimic stroke symptoms and subjects suffering from TIA or hemorrhagic stroke.

46. A method according to one of claims 34-40, wherein method provides an odds ratio of at least 2 for the prognostic risk of mortality.

47. A method according to one of claims 34-46, wherein the sample is selected from the group consisting of blood, serum, and plasma.

48. A method according to one of claims 34-47, wherein the cerebral injury is ischemic stroke.

49. A method according to one of claims 34-47, wherein the cerebral injury is hemorrhagic stroke.

50. A method according to one of claims 34-47, wherein the cerebral injury is traumatic brain injury.

51. A method for evaluating biomarker levels in a body fluid sample, comprising:

obtaining a body fluid sample from a subject selected for evaluation based on a determination that the subject is experiencing symptoms indicative of a possible ischemic stroke, hemorrhagic stroke, TIA, or traumatic brain injury and is in need of evaluation to determine the cause of such symptoms; and

performing one or more analyte binding assays configured to detect one or more biomarkers selected from the group consisting of eotaxin, epidermal growth factor receptor, S100A12, TIMP-4, and prolactin by introducing the body fluid sample obtained from the subject into an assay instrument which (i) contacts the body fluid sample with one or more binding reagents corresponding to the biomarker(s) being assayed, wherein each biomarker which is assayed binds to its respective specific binding reagent in an amount related to its concentration in the body fluid sample, (ii) generates one or more assay results indicative of binding of each biomarker which is assayed to its respective specific binding reagent; and (iii) displays the one or more assay results as a quantitative result in a human-readable form.

52. A method according to claim 51, wherein the assay result(s) are displayed as a concentration of each biomarker which is assayed.

53. A method according to claim 52, wherein the assay instrument further individually compares each biomarker concentration to a corresponding threshold level for that biomarker, and displays an indication of whether each biomarker does or does not exceed its corresponding threshold in a human-readable form.

54. A method according to claim 51, wherein a plurality of the biomarkers are measured, and wherein the assay results(s) comprise a single value calculated using a function that converts the concentration of each of the plurality of biomarkers into a single value.

55. A method according to claim 54, wherein the assay instrument further compares the single value to a corresponding threshold level and displays an indication of whether the single value does or does not exceed its corresponding threshold in a human-readable form.

56. A method for evaluating biomarker levels in a body fluid sample, comprising:

obtaining a body fluid sample from a subject selected for evaluation based on a determination that the subject has been diagnosed with an ischemic stroke, hemorrhagic stroke, TIA, or traumatic brain injury and is in need of risk stratification to determine a risl of one or more outcomes due to the ischemic stroke, hemorrhagic stroke, TIA, or traumatic brain injury; and

performing one or more analyte binding assays configured to detect one or more biomarkers selected from the group consisting of eotaxin, epidermal growth factor receptor, S100A12, TIMP-4, and prolactin by introducing the body fluid sample obtained from the subject into an assay instrument which (i) contacts the body fluid sample with one or more binding reagents corresponding to the biomarker(s) being assayed, wherein each biomarker which is assayed binds to its respective specific binding reagent in an amount related to its concentration in the body fluid sample, (ii) generates one or more assay results indicative of binding of each biomarker which is assayed to its respective specific binding reagent; and (iii) displays the one or more assay results as a quantitative result in a human-readable form.

57. A method according to claim 56, wherein the assay result(s) are displayed as a concentration of each biomarker which is assayed.

58. A method according to claim 57, wherein the assay instrument further individually compares each biomarker concentration to a corresponding threshold level for that biomarker, and displays an indication of whether each biomarker does or does not exceed its corresponding threshold in a human-readable form.

59. A method according to claim 56, wherein a plurality of the biomarkers are measured, and wherein the assay results(s) comprise a single value calculated using a function that converts the concentration of each of the plurality of biomarkers into a single value.

60. A method according to claim 59, wherein the assay instrument further compares the single value to a corresponding threshold level and displays an indication of whether the single value does or does not exceed its corresponding threshold in a human-readable form.

61. A method according to one of claims 56-60, wherein the subject is selected for evaluation of a mortality risk within a period selected from the group consisting of 21 days, 14 days, 7 days, 5 days, 96 hours, 72 hours, 48 hours, 36 hours, 24 hours, and 12 hours.

62. A method according to one of claims 51-61, wherein the plurality of assays are immunoassays performed by (i) introducing the body fluid sample into an assay device comprising a plurality of antibodies, at least one of which binds to each biomarker which is assayed, and (ii) generating an assay result indicative of binding of each biomarker to its respective antibody.

63. A method according to one of claims 34-62, further comprising treating the patient for ischemic stroke, hemorrhagic stroke, TIA, or traumatic brain injury based on the assay result(s) obtained

64. A system for evaluating biomarker levels, comprising:

a plurality of reagents which specifically bind for detection a plurality of biomarkers selected from the group consisting of eotaxin, epidermal growth factor receptor, S100A12, TIMP-4, and prolactin;

an assay instrument configured to (i) receive a body fluid sample, (ii) contact the plurality of reagents with the body fluid sample and (iii) generate and quantitatively display in human readable form one or more assay results indicative of binding of each biomarker which is assayed to a respective specific binding reagent in the plurality of reagents.

65. A system according to claim 64 wherein the reagents comprise a plurality of antibodies, at least one of which binds to each of the biomarkers which are assayed.

66. A system according to claim 65 wherein assay instrument comprises an assay device and an assay device reader, wherein the plurality of antibodies are immobilized at a plurality of predetermined locations within the assay device, wherein the assay device is configured to receive the body fluid sample such that the body fluid sample contacts the plurality of predetermined locations, and wherein the assay device reader interrogates the plurality of predetermined locations to generate the assay results.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/038067

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - G01N 33/53 (2013.01)
USPC - 436/501
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(8) - G01N 33/48, 33/53 (2013.01)
USPC - 435/7.1, 7.2, 7.94, 7.95; 436/501

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC - G01N 33/6896, 33/6893; G01N 2800/2871 (2013.01)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Orbit, Google Scholar, PubMed

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2008/0220013 A1 (HOCHSTRASSER et al) 11 September 2008 (11.09.2008) entire document	1, 2, 4-7, 18-20, 23-25, 31-33 --- 3, 21, 22, 26-28, 34-40, 51-61, 64-66
Y	WO 2011/075744 A1 (ANDERBERG et al) 23 June 2011 (23.06.2011) entire document	3, 21, 22, 26-28, 36, 54, 55, 59-61
Y	US 2009/0239241 A1 (RAY et al) 24 September 2009 (24.09.2009) entire document	34, 35, 37-40, 51-53, 56-58, 64-66
A	US 2005/0255484 A1 (VALKIRS et al) 17 November 2005 (17.11.2005) entire document	1-7, 18-28, 31-40, 51-61, 64-66

☐ Further documents are listed in the continuation of Box C. ☐

* 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
03 September 2013	18 SEP 2013
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2013/038067

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

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

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

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

3. ☒ Claims Nos.: 8-17, 29, 30, 41-50, 62, 63
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.