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(71) Applicants: **NANYANG TECHNOLOGICAL UNIVERSITY** [SG/SG]; 50 Nanyang Avenue, Singapore 639798 (SG). **NATIONAL UNIVERSITY OF SINGAPORE** [SG/SG]; 21 Lower Kent Ridge Road, Singapore 119077 (SG).

(72) Inventors: **SZE, Siu Kwan**; c/o NANYANG TECHNOLOGICAL UNIVERSITY, 50 Nanyang Avenue, Singapore 639798 (SG). **CHEN, Christopher**; c/o NATIONAL UNIVERSITY OF SINGAPORE, 21 Lower Kent Ridge Road, Singapore 119077 (SG). **DATTA, Arnab**; c/o NANYANG TECHNOLOGICAL UNIVERSITY, 50 Nanyang Avenue, Singapore 639798 (SG). **GALLART, Palau Xavier Ramon**; c/o NANYANG TECHNOLOGICAL UNIVERSITY, 50 Nanyang Avenue, Singapore 639798 (SG).

(74) Agent: **VIERING, JENTSCHURA & PARTNER LLP**; P.O. Box 1088, Rochor Post Office, Rochor Road, Singapore 911833 (SG).

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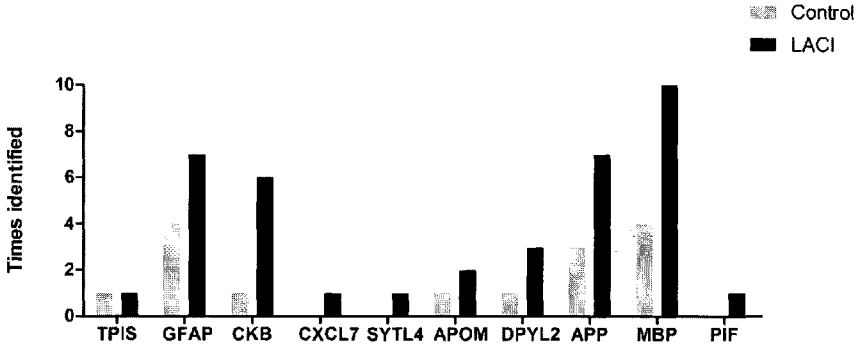
(54) Title: PLASMA MICROVESICLE BIOMARKERS FOR ISCHEMIC STROKE

Figure 15



(57) Abstract: The present invention relates to the identification of plasma microvesicle biomarkers for ischemic stroke and methods and uses thereof for diagnosing ischemic stroke and/or determining the prognosis of a subject suffering from ischemic stroke.

PLASMA MICROVESICLE BIOMARKERS FOR ISCHEMIC STROKE

Field of the Invention

[001] The present invention relates to the detection of plasma microvesicle biomarkers for ischemic stroke and methods and uses thereof for diagnosing ischemic stroke and/or determining the prognosis of a subject suffering from ischemic stroke.

Background of the Invention

[002] Stroke is one of the major causes of mortality, morbidity and disability. Stroke is a manifestation of vascular injury to the brain and responsible for more than 20% of all deaths worldwide (Lopez, A. D.; et al. Lancet 2006, 367, (9524), 1747-1757). In Singapore, stroke is a major cause of mortality and morbidity which accounts for more than 29% of the total years of life lost (Phua, H. P.; et al. Singapore Med J 2009, 50, (5), 468-78.). Stroke can be categorized into two broad types, "ischemic stroke" and "hemorrhagic stroke." Ischemic stroke encompasses thrombotic, embolic, lacunar and hypoperfusion types of strokes. Thrombolysis remains the only effective treatment strategy for acute ischemic stroke (Donnan, G. A.; et al. Nature Reviews Neurology 2011, 7, (7), 400-409).

[003] Lacunar infarction (LACI) is a subtype of ischemic stroke that accounts for approximately a quarter of all ischemic stroke cases (Wardlaw, J. M., *Future Neurology* **2011,** *6* (2), 201-221). Inadequacies of the imaging tools to reliably examine small cerebral arteries and arterioles cause potential misclassification of this ischemic stroke subtype for diagnosis, treatment and prognostication. Current stroke guidelines do not differentiate between lacunar and nonlacunar strokes (e.g. large vessel stroke or cardioembolic) with respect to treatment, risk factor modification or long-term outcome (Rajapakse, A.et al. *Stroke* **2011,** *42* (1), 217-220). Similarly, many of the major secondary stroke prevention trials have not distinguished between the different types of ischemic stroke, which may be important in determining the differential protective influence of various therapeutic approaches (e.g., antiplatelet drugs or thrombolysis) (Kirshner, H. S. *J. Neurol.* **2010,** *257* (11), 1788-1797). However, mounting evidence suggests differences in LACI pathology by comparison with non-lacunar strokes. Nevertheless, LACI remains a poorly understood area in terms of its etiology, pathophysiology, and more importantly, prognosis (De Silva, D.

A.; et al. *Journal of the Neurological Sciences* **2007,** *260* (1-2), 147-9.).

[004] Unlike non-lacunar subtypes of ischemic stroke, the short-term prognosis of ischemic cerebral small-vessel disease (SVD), including LACI is more favorable with an almost negligible early mortality, an absence of neuropsychological impairment and an excellent neurological recovery. However, LACI causes an increase in the mid- or long-term risk of recurrent vascular events and cognitive impairment or neuropsychological abnormalities. It has been shown recently that the proportion of dementia caused by SVD ranges from 36 to 67% (Grau-Olivares, M.; Arboix, A., *Expert Review of Neurotherapeutics* **2009,** *9* (8), 1201-1217). Therefore, apart from early diagnosis of LACI, identifying the patient cohorts that are at mid- or long-term risk for recurrent vascular events or complications, such as vascular dementia or neuropsychological abnormalities, will individualize and improve the treatment and recovery paradigms.

[005] Blood-based biomarkers can serve as an alternative tool to complement and improve the prognostic ability of clinical features and neuroimaging. Biomarkers for prognosis of ischemic stroke are a relatively new concept compared to other therapeutic areas such as cancer. No single or panel of blood-based biomarkers has been validated by clinical trials for stroke or related secondary complications. Therefore, CSF (Zimmermann-Ivol, C. G. et al. *Mol. Cell. Proteomics* **2004,** *3* (1), 66-72) or brain extracellular fluid (Giron, P.; et al. *Journal of Proteome Research* **2011,** *10* (1), 249-258) have been used as starting materials for biomarker discovery in stroke. Such biological samples are however painful and difficult to extract because of the extremely invasive nature of the methods used. To solve this several studies had been performed to validate protein biomarkers from blood (Foerch, C.; et al. *Neurology* **2009,** *73* (5), 393-9.), but only a few of these studies were directed specifically to SVD (Elkind, M. S. V.; et al. *International Journal of Stroke* **2010,** *5* (2), 117-125). In addition, most have tried to validate one or a few candidates (Bettermann, K., *J Stroke Cerebrovasc Dis* **2011,** *20* (3), 173-6.).

[006] Brain-specific Myelin basic protein (MBP) has been detected in the systemic circulation in nanogram concentration during the acute phase (e.g. hrs to few days) of ischemic stroke and is correlated with acute (24 hrs) or subacute (3 months) outcomes using targeted assays (Hill, M. D.; et al. *CMAJ* **2000,** *162* (8), 1139-40). Generally, any circulatory biomarkers present in the brain that may be associated with

neurological disorders would not be found in plasma as brain parenchyma remains selectively accessible by the systemic circulation due to the presence of blood brain barrier (BBB) under physiological conditions. However, different cell types in the brain (e.g. microglia and oligodendrocytes) are reported to release substances to the neighboring cells and external environment. A fraction of these substances may drain into the cerebrospinal fluid (CSF) and eventually in the blood. BBB abnormality is generally more diffuse in small vessel stroke compared to non-lacunar stroke subtypes that may cause gradual and sustained leakage of brain-specific MBP into general circulation (Wardlaw, J. M.; et al. *Stroke* **2003,** *34* (3), 806-12.). Chronic hypoperfusion of the white matter leading to progressive and selective death of oligodendrocytes by apoptosis and subsequent degeneration of myelinated fibres have been demonstrated in animals and in autopsized human brain of small vessel disease (Pantoni, L., *The Lancet Neurology* **2010,** *9* (7), 689-701).

[007] Using whole blood plasma limited the detection sensitivity for low abundant proteins. The proteomic approach for biomarker discovery from crude plasma is technically limited by its complexity and extreme dynamic range ($>10^{10}$), thereby resulting in poor sensitivity for detecting low abundant plasma proteins (Anderson, L.; Hunter, C. L., *Mol. Cell. Proteomics* **2006,** *5* (4), 573-588). To overcome this challenge, multiple approaches have been described that includes biophysical fractionation, enrichment of target sub-proteome and immuno-depletion of the most abundant interfering proteins. However, none of them were able to significantly outclass the other techniques (Whiteaker, J. R.; et al. *Journal of Proteome Research* **2007,** *6* (2), 828-836.).

[008] In view of the above, LACI remains a poorly understood area among acute ischemic stroke whose etiology, pathophysiology, and more importantly, the diagnosis and treatment largely remains in the shadow of non-lacunar subtypes despite it has relatively higher prevalence among Asian populations. Lack of well-defined diagnostic strategies and risk-factor based classifications are some of the major reasons for the above-mentioned shortcomings. Routinely used brain imaging techniques are often not sufficient to produce high resolution images for patients with LACI affecting small arterioles (0.2 – 0.8 mm) and having smaller sized infarcts than the larger territorial infarct seen in non-lacunar strokes. Neuroimaging techniques usually have low sensitivity to detect small infarcts in certain locations (posterior

fossa, cortex) and during the acute stage cannot differentiate between acute and old lesions. The diagnostic dilemma causes misclassification of approximately 20% of lacunar stroke as cortical stroke or vice-versa. In addition, there are situations where MRI is contraindicated, such as in medically unstable patients or those with an MRI-incompatible implanted device, body metal, or severe claustrophobia. Hence, an unmet need exists to complement and improve the effectiveness of the existing symptomatic and diagnostic protocols in the acute setting.

Summary of the Invention

[009] The present invention is based on the inventors' surprising finding that plasma microvesicles comprise biomarkers that are useful in the diagnosis of ischemic stroke and related conditions as well as determining the prognosis of stroke patients.

[0010] Microvesicles are membrane-bound vesicles, ranging in size from 0.04-1 μm. They are reported to be involved in the pathogenesis of various diseases such as ischemic stroke (Horstman, L. L.; et al. *Neurological Research* **2009,** *31* (8), 799-806), thrombosis, diabetes, inflammation, atherosclerosis and vascular cell proliferation (Azevedo, L. C. P.; et al. *Recent Patents on Cardiovascular Drug Discovery* **2007,** *2* (1), 41-51). Microvesicles can be secreted from endothelial, circulatory (e.g. platelets, leukocytes, erythrocytes), and even central nervous system (CNS)-specific cell types (e.g. microglia and oligodendrocytes) (Mathivanan, S.; Ji, H.; Simpson, R. J., *Journal of Proteomics* **2010,** *73* (10), 1907-20.).

[0011] Accordingly, a first aspect of the invention relates to a method for determining the prognosis of a subject afflicted by ischemic stroke comprising: a) isolating plasma microvesicles from a plasma sample obtained from the subject; and b) determining the presence and/or amount of at least one biomarker associated with the isolated plasma microvesicles; c) comparing the determined presence and/or amount of said at least one biomarker with a control, wherein a change in the presence and/or amount of the at least one biomarker relative to said control is indicative for said subject having an unfavorable prognosis.

[0012] A further aspect of the invention relates to a method for predicting the risk of a subject developing ischemic stroke, comprising: a) isolating plasma microvesicles from a plasma sample obtained from the subject; and b) determining the

presence and/or amount of at least one biomarker associated with the isolated plasma microvesicles; c) comparing the determined presence and/or amount of said at least one biomarker with a control, wherein a change in the presence and/or amount of the at least one biomarker relative to said control is indicative for an increased risk of said subject developing ischemic stroke.

[0013] A still further aspect of the invention relates to a method for diagnosing ischemic stroke in a subject comprising: a) isolating plasma microvesicles from a plasma sample obtained from the subject; and b) determining the presence and/or amount of at least one biomarker associated with the isolated plasma microvesicles; c) comparing the determined presence and/or amount of said at least one biomarker with a control, wherein a change in the presence and/or amount of the at least one biomarker relative to said control is indicative for said subject having suffered an ischemic stroke.

[0014] Another aspect of the invention relates to a panel of biomarkers for determining the prognosis of a subject afflicted by ischemic stroke, predicting the risk of a subject developing ischemic stroke or diagnosing ischemic stroke in a subject, wherein the panel comprises at least one, preferably at least two, more preferably at least 5 markers selected from the group consisting of polypeptides set forth in SEQ ID NOS 1-114.

[0015] In another aspect, the invention relates to use of the panel of biomarkers disclosed herein for determining the prognosis of a subject afflicted by ischemic stroke, predicting the risk of a subject developing ischemic stroke or diagnosing ischemic stroke in a subject.

[0016] Other aspects of the invention will be apparent to a person skilled in the art with reference to the following drawings and description of various non-limiting embodiments.

Brief Description of the Drawings

[0017] The drawings are not necessarily drawn to scale, emphasis instead generally being placed upon illustrating the principles of various embodiments. In the following description, various embodiments of the invention are described with reference to the following drawings.

[0018] **Figure 1.** Schematic representation of the experimental design. ERLIC,

electrostatic repulsion hydrophilic interaction chromatography.

[0019] **Figure 2**. Demographic Characteristics of the Patient Population Stratified by the Outcome Measures.

[0020] **Figure 3**. Pictures of a representative microvesicle pellet against different backgrounds. The loose hollow at the bottom of the tubes (shown by arrow) was obtained by sequential normal (4000g, 12000g) and ultra-centrifugation (30000g and 200000g) of plasma.

[0021] **Figure 4**. The List of Qualified and Regulated Plasma Microvesicle Proteome. The list contains quantitative information of the selected proteins from bias and background corrected iTRAQ data set. The denominator is the demographically matched control. Unused and % coverage are parameters related to the confident identification of proteins. This list contains 43 candidates qualified (out of 183) through the initial filters [i.e., unused prot score >3.0 and FDR = 1.1% (confident identification), p-value <0.05 (significantly different from 1) for at least two ratios]. The significant ratios are indicated in bold. The Uniport accession numbers of the 'unreviewed' proteins are indicated in italics form. The protein whose evidence is available only at the level of transcript is not provided with a gene symbol. The last column provides information about GO or pathway. CCC = complement and coagulation cascade, ECS = extracellular space, EIA = enzyme inhibitor activity, FA = focal adhesion, IIR = innate immune response, LT= lipid transport, RTW = response to wounding.

[0022] **Figure 5. A** List of the five most significant up-regulated biomarkers in plasma microvesicles of LACI patient with adverse outcome, and **B** List of the five most significant down-regulated biomarkers in plasma microvesicles of LACI patient with adverse outcome.

[0023] **Figure 6.** List of unique up-regulated biomarkers in plasma microvesicles of LACI patient with adverse outcome.

[0024] **Figure 7.** List of unique down-regulated biomarkers in plasma microvesicles of LACI patient with adverse outcome.

[0025] **Figure 8**. **A** Histogram showing the iTRAQ ratios of selected proteins related to focal adhesion (ITGA2B, TLN1 and FLNA) and coagulation cascade (FGA, FGB and PLG). Demographically matched control group was used as the common denominator (i.e. 114) for comparing the three groups of LACI patients. The solid line

indicates no change in regulation. The LACI groups with adverse outcome (recurrent vascular event, 116; cognitive decline, 117) had a differential signature in comparison with the LACI group with no adverse outcome (i.e. 115). Up-regulation of proteins related to focal adhesion and coagulation (FGA, FGB) is predictive of poor outcome. * Denotes ratios with significant p-value (< 0.05). **B** Technical validation of iTRAQ result by Western Blot analysis of ALB on pooled lysates. ALB showed downward trend in LACI – recurrent vascular event group unlike the LACI group with no adverse outcome, which is consistent with the iTRAQ result.

[0026] **Figure 9**. Synopsis of the in vivo study to capture the interplay of multiple events in the ischemic brain by iTRAQ-MuDPIT approach.

[0027] **Figure 10**. Schematic diagram showing the 4-plex iTRAQ-MuDPIT guided biomarker discovery approach.

[0028] **Figure 11**. Schematic diagram showing the experimental design. Shortlisted candidates from the discovery phase will be validated by duel strategy of multiplex validation involving Luminex and MRM assay. Fifty patients with LDV (large vessel disease) will be included in two phases to check the specificity of the candidate biomarkers. **Number of LACI patients in different sub-groups is variable.

[0029] **Figure 12. A** Western blot of MBP in control samples and samples taken from LACI patents that exhibit recurrent vascular events and **B** densitometry results of the western blots.

[0030] **Figure 13. A** MS/MS quantification of MBP from microvesicles isolated from 500µl of plasma using a combination of a sucrose gradient and ultracentrifugation, and **B** the western blot quantification of MBP from microvesicles isolated from 500µl of plasma using a combination of a sucrose gradient and ultracentrifugation.

[0031] **Figure 14.** Western blot quantification of MBP from microvesicles isolated from 100µl of plasma using soluble protein precipitation for microvesicle purification method.

[0032] **Figure 15.** Bar graph showing the number of times that each LACI biomarker has been identified in the plasma exosomal fraction of individual subjects (healthy control and LACI patients including recurrent vascular events and cognitive decline) by LC-MS/MS. The isolation of the exosomal fraction was done following

the "soluble protein precipitation for microvesicle purification method" described in the example 20.

Detailed Description

[0033] Considering the research gaps stated in the background section above, blood-based biomarkers were developed as an alternative tool to complement and improve the effectiveness of the existing protocols relying on clinical features and neuroimaging. By predicting LACI patients who are at risk of recurrent vascular events or cognitive decline, these biomarkers can guide clinicians to stratify patients for testing therapeutic options or rehabilitation schemes in clinical trials and practice. Conversely, these biomarkers can also be used as surrogate markers in LACI related clinical trials to monitor the consequences of therapeutic interventions. The detailed profiling of the plasma samples performed during the discovery phase and the proposed validation of a panel of proteins could also facilitate better understanding of the underlying pathology and stimulate research interest on individual biomarkers in the absence of a comparable dataset from autopsied LACI affected brain samples.

[0034] To mitigate the challenge of using whole plasma, microvesicles were isolated from plasma for stroke prognostic biomarker identification. An iTRAQ-2D-LC-MS/MS-guided shotgun proteomic strategy was applied for the discovery of potential prognostic biomarker of LACI by comparative profiling of plasma microvesicles in three groups of LACI patients and a group of demographically-matched control. Plasma samples of forty five LACI patients from the European Australasian Stroke Prevention in Reversible Ischemia Trial (ESPRIT) were used for the study (De Schryver, E. L. L. M., *Cerebrovasc. Dis.* **2000,** *10* (2), 147-150). The patients were monitored for up to 5 yrs after index stroke for adverse outcomes (i.e. recurrent vascular events or decline in cognitive functions). Microvesicles were collected for the iTRAQ experiment.

[0035] Analysis of the significantly changed protein levels from the iTRAQ data set revealed up-regulation of a group of proteins from brain tissue in the plasma microvesicles, and proteins related to the integrin signaling (e.g. ITGA2B, TLN1, and FLNA) and coagulation cascade (FGA, FGB) that are associated with an unfavorable outcome. Given that blood collection is a simple and cheap procedure; these biomarkers can have additive value over the existing imaging, clinical or

neurobehavioral modalities used in the clinic for diagnosis and prognosis of stroke, particularly for asymptomatic/silent stroke, LACI and transient ischemic attack (TIA) stroke subtypes.

[0036] Accordingly, a first aspect of the invention relates to a method for determining the prognosis of a subject afflicted by ischemic stroke comprising: a) isolating plasma microvesicles from a plasma sample obtained from the subject; and b) determining the presence and/or amount of at least one biomarker associated with the isolated plasma microvesicles; c) comparing the determined presence and/or amount of said at least one biomarker with a control, wherein a change in the presence and/or amount of the at least one biomarker relative to said control - that exceeds a given threshold value - is indicative for said subject having an unfavorable prognosis.

[0037] For purposes of the following discussion, the methods described as applicable to the prognosis of a subject afflicted by ischemic stroke generally may be considered applicable to an individual that has had an ischemic stroke including thrombotic, embolic and/or lacunar stroke and/or hypoperfusion. Preferably, the ischemic stroke is selected from the group consisting of lacunar infarction (LACI), transient ischemic attack (TIA) or silent stroke, most preferably the ischemic stroke is a lacunar infarction.

[0038] In addition, while the following embodiments are described in relation to the prognostic method of the invention, it is understood that many embodiments are equally applicable in the other methods of the invention, for example the methods of diagnosis or monitoring therapy.

[0039] The prognosis is intended to determine the potential of the individual to have an adverse outcome. An adverse outcome may include a recurrent vascular event or cognitive decline.

[0040] The term "prognosis" as used herein refers to methods by which the person skilled in the art such as a health professional can predict the course or outcome of a condition in a patient. The term "prognosis" does not refer to the ability to predict the course or outcome of a condition with 100% accuracy, or even that a given course or outcome is more likely to occur than not. Instead, the skilled health professional will understand that the term "prognosis" refers to an increased probability that a certain course or outcome will occur; that is, that a course or outcome is more likely to occur in a subject exhibiting a given indicator, when

compared to those individuals not exhibiting the indicator.

[0041] The skilled professional will understand that associating a prognostic indicator with a predisposition to an adverse outcome is a statistical analysis. For example, a biomarker amount isolated from plasma microvesicles that varies from the amount of said biomarker in a control by at least 10% may indicate that the subject is more likely to suffer from an adverse outcome than patients with a level that varies less than or equal to 10%, as determined by a level of statistical significance.

[0042] Additionally, a change in marker concentration from baseline levels may be reflective of patient prognosis, and the degree of change in biomarker level may be related to the severity of adverse events. Statistical significance is often determined by comparing two or more populations, and determining a confidence interval and or a p value. Preferred confidence intervals of the invention are 90%, 95%, 97.5%, 98%, 99%, 99.5%, 99.9% and 99.99%, while preferred p values are 0.1, 0.05, 0.025, 0.02, 0.01, 0.005, 0.001, and 0.0001. Exemplary statistical tests for associating a prognostic indicator with a predisposition to an adverse outcome are described hereinafter.

[0043] In various embodiments, a threshold degree of change in the level of a prognostic or diagnostic indicator can be established, and the degree of change in the level of the indicator in a subject sample can simply be compared to the threshold degree of change in the level. A preferred threshold change in the level for markers of the invention is about 5%, about 10%, about 15%, about 20%, about 25%, about 30%, about 50%, about 75%, about 100%, and about 150%, always in relation to a control value. The term "about" in this context refers to +/- 3%.

[0044] It should however be understood that instead of comparing a determined marker value with a control, an absolute threshold value can be determined based on previously determined control values, wherein if the determined marker amount exceeds or falls below (depending on whether up- or down-regulation of the marker is found significant) the given threshold, the change is considered relevant for prognosis.

[0045] As used herein the term “plasma microvesicles” refer to plasma membrane fragments, typically small membrane vesicles of between 0.03 and 1 micrometer (μm) in diameter. They are formed by cells through a process of membrane budding or exocytosis. The term as used herein also includes exosomes. Exosomes are cell-derived vesicles that are smaller than about 150 nm in diameter,

typically with diameters between 30 and 100 nm.

[0046] According to the described methods, the plasma microvesicles are isolated from a plasma sample.

[0047] As used herein "plasma" refers to the liquid component of whole blood that makes up approximately 55% of the total blood volume.

[0048] Generally a blood sample may be drawn from a vein and the plasma subsequently separated from the cellular blood components. However, any method of extracting blood known in the art would be similarly suitable. There are many methods known to those in the art for separating plasma from blood and any such method would be suitable for application of the present methods. The amount of blood that needs to be obtained from an individual may depend on the desired sample size and is well known to those skilled in the art.

[0049] The plasma microvesicles are typically isolated from the plasma sample by ultracentrifugation, sucrose gradient density centrifugation or soluble protein precipitation for microvesicle purification method where soluble proteins are precipitated by a suitable method such as organic solvent precipitation using acetone, methanol and acetonitrile and other suitable solvents.

[0050] The term "biomarker" as used herein refers to proteins or polypeptides to be used as targets for screening test samples obtained from the isolated plasma microvesicles of the subjects. "Proteins or polypeptides" used as biomarkers in the present invention are contemplated to include any fragments thereof, in particular, immunologically detectable fragments.

[0051] In various embodiments the biomarker is detected using a suitable detection reagent. The type of said reagent is dependent on the type of biomarker to be detected. In case the biomarker is a protein, polypeptide or peptide, the detection reagent may, for example, be an antibody or antibody-like molecule or known derivatives thereof. In various embodiments, the detection reagents may also include conjugates of the respective binding moeity, e.g. the antibody, that facilitate detection, such as biotin or fluorescent labels or artificial sequence tags. Such conjugates may be non-natural variants of the relevant binding part of the detection reagent.

[0052] "At least one", as used herein, in particular in relation to the biomarkers, may include a single one, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more. In certain embodiments, the inventive methods employ the

detection/determination of at least two biomarkers, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 12, at least 14, at least 15, or at least 20 biomarkers.

[0053] The term "subject afflicted by ischemic stroke" as used herein refers to an individual that has or is suspected to have suffered an ischemic stroke within the last 6 months or less. Generally, as already mentioned above an ischemic stroke includes thrombotic, embolic and/or lacunar stroke and hypoperfusion. Preferably, the ischemic stroke is selected from the group consisting of lacunar infarction (LACI), transient ischemic attack (TIA) or silent stroke, most preferably the ischemic stroke is a lacunar infarction. The terms "lacunar stroke" and "lacunar infarction" as used interchangeably herein and relate to a type of stroke that results from occlusion of one of the penetrating arteries that provides blood to the brain's deep structures.

[0054] In various embodiments the unfavorable prognosis is an increased likelihood for an adverse event selected from the group of developing cognitive impairment, including dementia, and/or recurrent vascular events. The adverse event may also include reduced survival and death.

[0055] As used herein, the term "recurrent vascular events" may include another ischemic stroke, as defined above, in particular an ischemic event selected from lacunar infarction (LACI), transient ischemic attack (TIA) and silent stroke.

[0056] As used herein, the term "increased likelihood" relates to the increased chance of an individual to develop an adverse event compared to a control subject, e.g. a normal healthy subject or a subject that does not exhibit a significant change in one or more of the assessed biomarker levels.

[0057] In various embodiments, the prognosis is a long-term prognosis, preferably a prognosis for a time period of more than 12, more than 24, more than 36, more than 48, or more than 60 months.

[0058] A further aspect of the invention relates to a method for predicting the risk of a subject developing ischemic stroke comprising: a) isolating plasma microvesicles from a plasma sample obtained from the subject; and b) determining the presence and/or amount of at least one biomarker associated with the isolated plasma microvesicles and or exosomes; c) comparing the determined presence and/or amount of said at least one biomarker with a control, wherein a change in the presence and/or amount of the at least one biomarker relative to said control - that exceeds a given

threshold value - is indicative for an increased risk of said subject developing ischemic stroke.

[0059] The term "predicting the risk of a subject developing ischemic stroke" refers to determining the likelihood that a subject that has never suffered from an ischemic stroke will suffer ischemic stroke. The ischemic stroke may be a thrombotic, embolic or lacunar stroke or hypoperfusion. Preferably, the ischemic stroke is selected from the group consisting of lacunar infarction (LACI), transient ischemic attack (TIA) or silent stroke, most preferably the ischemic stroke is a lacunar infarction.

[0060] The prediction is by a statistical determination of the statistical chance of the individual's predisposition to suffer an ischemic stroke.

[0061] A further aspect of the invention relates to a method for diagnosing ischemic stroke in a subject comprising: a) isolating plasma microvesicles from a plasma sample obtained from the subject; and b) determining the presence and/or amount of at least one biomarker associated with the isolated plasma microvesicles and or exosomes; c) comparing the determined presence and/or amount of said at least one biomarker with a control, wherein a change in the presence and/or amount of the at least one biomarker relative to said control - that exceeds a given threshold value - is indicative for said subject having suffered an ischemic stroke.

[0062] The term "diagnosing" as used herein refers to methods by which the skilled health professional can estimate and/or determine whether or not a patient is suffering from a given disease or condition. The skilled professional often makes a diagnosis on the basis of one or more diagnostic indicators, i.e., a biomarker, the presence, absence, or amount of which is indicative of the presence, severity, or absence of the condition. Ischemic stroke includes the subtypes listed above.

[0063] In various embodiments of the described methods of diagnosis or risk determination, the subject has suffered suspected or confirmed lacunar infarction.

[0064] In various embodiments of all the above-described methods, the subject is diagnosed with or already undergoing a therapy for ischemic cerebral small vessel disease (CVD).

[0065] In various embodiments, the control is a control subject, in particular a subject who has not suffered ischemic stroke or the relevant stroke subtype.

[0066] In various embodiments the plasma microvesicles include or are exosomes. The method may thus include the step of isolating the exosomes from a

patient's sample.

[0067] In various embodiments the plasma microvesicles, including the exosomes, are isolated by ultracentrifugation. In various alternative embodiments, the plasma microvesicles are isolated by sucrose density gradient centrifugation. Such an isolation method has the advantage of requiring a smaller volume of plasma, for example 500µl or less, to obtain a sufficient amount of plasma microvesicles for biomarker analysis. In various embodiments, the plasma microvesicles are isolated by soluble protein precipitation for microvesicle purification method. Such an isolation method has the advantage of requiring an even smaller volume of plasma, such as 100µl or less, to obtain a sufficient amount of plasma microvesicles for biomarker analysis. In addition, the latter method has the further advantage of allowing isolating the plasma microvesicles in about 1 hour which saves time and thus allows faster results of disease prognosis or diagnosis.

[0068] In various embodiments the amount of the at least one biomarker is determined by a combined chromatography/mass spectrometry technique, preferably LC/MS/MS technique, more preferably iTRAQ (isobaric tags for relative and absolute quantification). However, the skilled person will readily understand that in principle any proteomics methods known in the art may be similarly used.

[0069] In various embodiments the one or more biomarkers are selected from the group consisting of the polypeptides having the amino acid sequences set forth in SEQ ID Nos. 1-114.

[0070] In various embodiments the one or more biomarkers are selected from the group consisting of the polypeptides: Alpha-2-macroglobulin (A2M) that is upregulated in patients with recurrent vascular events and upregulated in patients with cognitive decline. In preferred embodiments A2M has Uniprot accession number P01023 (Version 3), for example having the amino acid sequence as set forth in SEQ ID NO. 1; Complement C3 (C3) that is downregulated in patients with recurrent vascular events and downregulated in patients with cognitive decline. In preferred embodiments C3 has Uniprot accession number P01024 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 2; Serum albumin (ALB) that is downregulated in patients with recurrent vascular events and downregulated in patients with cognitive decline. In preferred embodiments ALB has Uniprot accession number P02768 (Version 2), for example having the amino acid sequence as set forth

in SEQ ID NO. 3; Fibrinogen alpha chain (FGA) that is upregulated in patients with recurrent vascular events and upregulated in patients with cognitive decline. In preferred embodiments FGA has Uniprot accession number P02671 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 4; Fibrinogen beta chain (FGB) that is upregulated in patients with recurrent vascular events and upregulated in patients with cognitive decline. In preferred embodiments FGB has Uniprot accession number P02675 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 5; Haptoglobin (HP) that is downregulated in patients with recurrent vascular events and downregulated in patients with cognitive decline. In preferred embodiments HP has Uniprot accession number P00738 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 6; Fibrinogen gamma chain (FGG) that is upregulated in patients with recurrent vascular events and upregulated in patients with cognitive decline. In preferred embodiments FGG has Uniprot accession number P02679 (Version 3), for example having the amino acid sequence as set forth in SEQ ID NO. 7; von Willebrand factor (VWF) that is upregulated in patients with recurrent vascular events and upregulated in patients with cognitive decline. In preferred embodiments VWF has Uniprot accession number P04275, (Version 4), for example having the amino acid sequence as set forth in SEQ ID NO. 8; Complement C5 (C5) that is downregulated in patients with recurrent vascular events and downregulated in patients with cognitive decline. In preferred embodiments C5 has Uniprot accession number P01031, (Version 4), for example having the amino acid sequence as set forth in SEQ ID NO. 9; IgGFc-binding protein (FCGBP) that is upregulated in patients with recurrent vascular events and upregulated in patients with cognitive decline. In preferred embodiments FCGBP has Uniprot accession number Q9Y6R7 (Version 3), for example having the amino acid sequence as set forth in SEQ ID NO. 10; Complement component 4 binding protein, alpha (C4BPA) that is upregulated in patients with recurrent vascular events and downregulated in patients with cognitive decline. In preferred embodiments C4BPA has Uniprot accession number Q5VVQ8 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 11; Apolipoprotein A-1 (APOA1) that is downregulated in patients with recurrent vascular events and downregulated in patients with cognitive decline. In preferred embodiments APOA1 has Uniprot accession number P02647 (Version 1), for example having the amino acid sequence

as set forth in SEQ ID NO. 12; Galectin-3-binding protein (LGALS3BP) that is downregulated in patients with recurrent vascular events and upregulated in patients with cognitive decline. In preferred embodiments LGALS3BP has Uniprot accession number Q08380 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 13; Non-muscle myosin heavy polypeptide 9 (MYH9) that is upregulated in patients with recurrent vascular events and upregulated in patients with cognitive decline. In preferred embodiments MYH9 has Uniprot accession number Q60FE2 (Version 4), for example having the amino acid sequence as set forth in SEQ ID NO. 14; Filamin-A (FLNA) that is upregulated in patients with recurrent vascular events and upregulated in patients with cognitive decline. In preferred embodiments FLNA has Uniprot accession number P21333 (Version 4), for example having the amino acid sequence as set forth in SEQ ID NO. 15; Tailin-1 (TLN1) that is upregulated in patients with recurrent vascular events and upregulated in patients with cognitive decline. In preferred embodiments TLN1 has Uniprot accession number Q9Y490 (Version 3), for example having the amino acid sequence as set forth in SEQ ID NO. 16; Hemoglobin subunit alpha (HBA1) that is downregulated in patients with recurrent vascular events and downregulated in patients with cognitive decline. In preferred embodiments HBA1 has Uniprot accession number P69905 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 17; CD5 antigen-like (CD5L) that is upregulated in patients with recurrent vascular events and upregulated in patients with cognitive decline. In preferred embodiments CD5L has Uniprot accession number O43866 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 18; Alpha-1-antitrypsin (SERPINA1) that is downregulated in patients with recurrent vascular events and downregulated in patients with cognitive decline. In preferred embodiments SERPINA1 has Uniprot accession number P01009 (Version 3), for example having the amino acid sequence as set forth in SEQ ID NO. 19; Plasminogen (PLG) that is downregulated in patients with recurrent vascular events and downregulated in patients with cognitive decline. In preferred embodiments PLG has Uniprot accession number P00747 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 20; Kallikrein B, plasma (Flecher factor) 1, isoform CRA_b (KLKB1) that is downregulated in patients with recurrent vascular events and upregulated in patients with cognitive decline. In preferred embodiments KLKB1 has Uniprot accession

number Q4W5C3 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 21; Vitamin K-dependent protein S (PROS1) that is downregulated in patients with recurrent vascular events and downregulated in patients with cognitive decline. In preferred embodiments PROS1 has Uniprot accession number P07225 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 22; Inter-alpha (Globulin) inhibitor H4 (plasma Kalikrein-sesitive glycoprotein (ITIH4) that is downregulated in patients with recurrent vascular eventsand downregulated in patients with cognitive decline. In preferred embodiments ITIH4 has Uniprot accession number B2RMS9 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 23; Apolipoprotein L1 (APOL1) that is downregulated in patients with recurrent LACI and upregulated in patients with cognitive decline. In preferred embodiments APOL1 has Uniprot accession number O14791 (Version 5), for example having the amino acid sequence as set forth in SEQ ID NO. 24; Lipoprotein Lp(A) (LPA) that is upregulated in patients with recurrent LACI and upregulated in patients with cognitive decline. In preferred embodiments LPA has Uniprot accession number Q1HP67 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 25; Hemopexin (HPX) that is downregulated in patients with recurrent LACI and downregulated in patients with cognitive decline. In preferred embodiments HPX has Uniprot accession number P02790 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 26; Apilipoprotein E (APOE) that is downregulated in patients with recurrent LACI and downregulated in patients with cognitive decline. In preferred embodiments APOE has Uniprot accession number P02649 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 27; Integrin alpha-IIb (ITGA2B) that is upregulated in patients with recurrent LACI and upregulated in patients with cognitive decline. In preferred embodiments ITGA2B has Uniprot accession number P08514 (Version 3), for example having the amino acid sequence as set forth in SEQ ID NO. 28; Immunoglobulin J chain (IGJ) that is upregulated in patients with recurrent LACI and upregulated in patients with cognitive decline. In preferred embodiments IGJ has Uniprot accession number P01591 (Version 4), for example having the amino acid sequence as set forth in SEQ ID NO. 29; Serum paraoxonase/arylesterase 1 (PON1) that is downregulated in patients with recurrent LACI and downregulated in patients with cognitive decline. In preferred

embodiments PON1 has Uniprot accession number P27169 (Version 3), for example having the amino acid sequence as set forth in SEQ ID NO. 30; Myelin basic protein (MBP) that is upregulated in patients with recurrent LACI and upregulated in patients with cognitive decline. In preferred embodiments MBP has Uniprot accession number P02686 (Version 3), for example having the amino acid sequence as set forth in SEQ ID NO. 31; Serine/cysteine proteiase inhibitor clade G member 1 splice variant 2 (fragment) (SERPING1) that is downregulated in patients with recurrent LACI and downregulated in patients with cognitive decline. In preferred embodiments SERPING1 has Uniprot accession number Q5UGI6 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 32; Apolipoprotein A-II (APOA2) that is downregulated in patients with recurrent LACI and upregulated in patients with cognitive decline. In preferred embodiments APOA2 has Uniprot accession number P02652 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 33; putative protein cDNA FLJ76826, highly similar to Homo sapiens ceruloplasmin (ferroxidase) (CP), mRNA that is downregulated in patients with recurrent LACI and downregulated in patients with cognitive decline. In preferred embodiments the putative protein has Uniprot accession number A8K5A4 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 34; putative protein cDNA, FLJ94213, highly similar to Homo sapiens pregnancy-zone protein (PZP), mRNA that is downregulated in patients with recurrent LACI and upregulated in patients with cognitive decline. In preferred embodiments the putative protein has Uniprot accession number B2R950 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 35; putative protein cDNA FLJ75066, highly similar to Homo sapiens complement component 1, r subcomponent (C1R), mRNA that is downregulated in patients with recurrent LACI and downregulated in patients with cognitive decline. In preferred embodiments the putative protein has Uniprot accession number A8K5J8 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 36; putative protein cDNA FLJ55673, highly similar to Complement factor B (EC 3.4.21.47) that is downregulated in patients with recurrent LACI and downregulated in patients with cognitive decline. In preferred embodiments the putative protein has Uniprot accession number B4E1Z4 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 37; putative uncharacterized protein (Fragment) that is

upregulated in patients with recurrent LACI and upregulated in patients with cognitive decline. In preferred embodiments the putative protein has Uniprot accession number Q8WVW5 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 38; putative protein Thrombospondin 1 variant (Fragment) that is upregulated in patients with recurrent LACI and upregulated in patients with cognitive decline. In preferred embodiments the putative protein has Uniprot accession number Q59E99 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 39; putative protein cDNA FLJ35730 fis, clone TESTI2003131, highly similar to ALPHA-1-ANTICHYMOTRYPSIN that is downregulated in patients with recurrent LACI and downregulated in patients with cognitive decline. In preferred embodiments the putative protein has Uniprot accession number B3KS79 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 40; cDNA FLJ59731, highly similar to complement component C8 beta chain (C8B) that is downregulated in patients with recurrent LACI and downregulated in patients with cognitive decline. In preferred embodiments C8B has Uniprot accession number B7Z550 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 41; putative protein Peptidyl-prolyl cis-trans isomerase that is upregulated in patients with recurrent LACI and upregulated in patients with cognitive decline. In preferred embodiments the putative protein has Uniprot accession number A8K486 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 42; putative protein Dihydropyrimidinase-like 2 variant (Fragment) that is upregulated in patients with recurrent LACI and upregulated in patients with cognitive decline. In preferred embodiments the putative protein has Uniprot accession number Q59GB4 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 43; Cholinesterase (BCHE) that is upregulated in patients with an adverse outcome. In preferred embodiments BCHE has Uniprot accession number P06276 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 44; Diydropyrimidinase-related protein 2 (DPYSL2) that is upregulated in patients with an adverse outcome. In preferred embodiments DPYSL2 has Uniprot accession number Q16555 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 45; Glial fibrillary acidic protein (GFAP) that is upregulated in patients with an adverse outcome. In preferred embodiments GFAP has Uniprot

accession number P14136 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 46; Dermcidin (PIF) that is upregulated in patients with an adverse outcome. In preferred embodiments PIF has Uniprot accession number Q53YJ2 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 47; Fermitin family homolog 3 (FERMT3) that is downregulated in patients with an adverse outcome. In preferred embodiments FERMT3 has Uniprot accession number Q86UX7 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 48; Multimerin-1 (MMRN1) that is downregulated in patients with an adverse outcome. In preferred embodiments MMRN1 has Uniprot accession number Q13201 (Version 3), for example having the amino acid sequence as set forth in SEQ ID NO. 49; Protein disulfide-isomerase (PDIA3) that is downregulated in patients with an adverse outcome. In preferred embodiments PDIA3 has Uniprot accession number P30101 (Version 4), for example having the amino acid sequence as set forth in SEQ ID NO. 50; Extended synaptotagmin-1 (ESYT1) that is downregulated in patients with an adverse outcome. In preferred embodiments ESYT1 has Uniprot accession number Q9BSJ8 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 51; Peptidyl-prolyl cis-trans isomerise A (PPIA) that is upregulated in patients with an adverse outcome. In preferred embodiments PPIA has Uniprot accession number P62937 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 52; Haptoglobin-related protein (HPR) that is upregulated in patients with an adverse outcome. In preferred embodiments HPR has Uniprot accession number P00739 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 53; Berta-globin (HBB) that is upregulated in patients with an adverse outcome. In preferred embodiments HBB has Uniprot accession number C8C504 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 54; Delta globin (fragment) (HBD) that is upregulated in patients with an adverse outcome. In preferred embodiments HBD has Uniprot accession number D1MGP8 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 55; Proline-rich protein 4 (PRR4) that is upregulated in patients with an adverse outcome. In preferred embodiments PRR4 has Uniprot accession number Q16378 (Version 3), for example having the amino acid sequence as set forth in SEQ ID NO. 56; Ferritin (FTH1) that is upregulated in patients with an adverse outcome. In preferred

embodiments FTH1 has Uniprot accession number Q6NZ44 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 57; APOL1 protein fragment (APOL1) that is upregulated in patients with an adverse outcome. In preferred embodiments APOL1 has Uniprot accession number A5PL32 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 58; PRSS1 protein (PRSS1) that is upregulated in patients with an adverse outcome. In preferred embodiments PRSS1 has Uniprot accession number Q3SY19 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 59; Creatine kinase B-type (CKB) that is upregulated in patients with an adverse outcome. In preferred embodiments CKB has Uniprot accession number P12277 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 60; Triosephosphate isomerise (TPI1) that is upregulated in patients with an adverse outcome. In preferred embodiments TPI1 has Uniprot accession number P60174 (Version 3), for example having the amino acid sequence as set forth in SEQ ID NO. 61; Putative uncharacterized protein PZP (PZP) that is upregulated in patients with an adverse outcome. In preferred embodiments PZP has Uniprot accession number A6ND27 (Version 4), for example having the amino acid sequence as set forth in SEQ ID NO. 62; Alpha-crystallin B chain (CRYAB) that is upregulated in patients with an adverse outcome. In preferred embodiments CRYAB has Uniprot accession number P02511 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 63; Cytochrome b-c1 complex subunit 6, mitochondrial (UQCRH) that is upregulated in patients with an adverse outcome. In preferred embodiments UQCRH has Uniprot accession number P07919 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 64; Calcium/calmodulin-dependent protein kinase II alpha (CAMK2A) that is upregulated in patients with an adverse outcome. In preferred embodiments CAMK2A has Uniprot accession number Q8IWE0 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 65; Ferritin (FTL) that is upregulated in patients with an adverse outcome. In preferred embodiments FTL has Uniprot accession number Q6IBT7 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 66; Cartilige oligomeric matrix protein (COMP) that is upregulated in patients with an adverse outcome. In preferred embodiments COMP has Uniprot accession number P49747 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO.

67; putative protein S that is upregulated in patients with an adverse outcome. In preferred embodiments the putative protein S has Uniprot accession number Q9NSD0 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 68; Ceruloplasmin (CP) that is upregulated in patients with an adverse outcome. In preferred embodiments CP has Uniprot accession number P00450 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 69; Putative uncharacterized protein RELN (RELN) that is upregulated in patients with an adverse outcome. In preferred embodiments RELN has Uniprot accession number C9J2G2, version 5 represented by SEQ ID NO. 70; Cytochrome c oxyidase subunit 6A1, mitochondrial (COX6A1) that is upregulated in patients with an adverse outcome. In preferred embodiments COX6A1 has Uniprot accession number P12074 (Version 4), for example having the amino acid sequence as set forth in SEQ ID NO. 71; COL6A3 protein (COL6A3) that is upregulated in patients with an adverse outcome. In preferred embodiments COL6A3 has Uniprot accession number B7ZW00 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 72; Amyloid protein A (APP) that is upregulated in patients with an adverse outcome. In preferred embodiments APP has Uniprot accession number B2R5G8 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 73; Apolipoprotein M (APOM) that is upregulated in patients with an adverse outcome. In preferred embodiments APOM has Uniprot accession number Q5SRP5 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 74; Vinculin (VCL) that is downregulated in patients with an adverse outcome. In preferred embodiments VCL has Uniprot accession number P18206 (Version 4), for example having the amino acid sequence as set forth in SEQ ID NO. 75; Integrin beta-3 (ITGB3) that is downregulated in patients with an adverse outcome. In preferred embodiments ITGB3 has Uniprot accession number P05106 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 76; Platelet glycoprotein Ib alpha polypeptide (GP1BA) that is downregulated in patients with an adverse outcome. In preferred embodiments GP1BA has Uniprot accession number A5CKE2, version 1 represented by SEQ ID NO. 77; ATP Synthase subunit beta, mitochondrial (ATP5B) that is downregulated in patients with an adverse outcome. In preferred embodiments ATP5B has Uniprot accession number P06576 (Version 3), for example having the amino acid sequence as set forth in SEQ ID NO. 78; Platelet

glycoprotein V (GP5) that is downregulated in patients with an adverse outcome. In preferred embodiments GP5 has Uniprot accession number P40197 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 79; Putative uncharacterized protein ITGA6 (ITGA6) that is downregulated in patients with an adverse outcome. In preferred embodiments ITGA6 has Uniprot accession number C9JK10 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 80; Band 3 anion transport protein (SLC4A1) that is downregulated in patients with an adverse outcome. In preferred embodiments SLC4A1 has Uniprot accession number P02730 (Version 3), for example having the amino acid sequence as set forth in SEQ ID NO. 81; Integrin-linked protein kinase (ILK) that is downregulated in patients with an adverse outcome. In preferred embodiments ILK has Uniprot accession number Q13418 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 82; Sarcoplasmic/endoplasmic reticulum calcium APTase 3 (ATP2A3) that is downregulated in patients with an adverse outcome. In preferred embodiments ATP2A3 has Uniprot accession number Q93084 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 83; Apolipoprotein A-IV (APOA4) that is downregulated in patients with an adverse outcome. In preferred embodiments APOA4 has Uniprot accession number P06727 (Version 3), for example having the amino acid sequence as set forth in SEQ ID NO. 84; Integrin alpha-2 (ITGA2) that is downregulated in patients with an adverse outcome. In preferred embodiments ITGA2 has Uniprot accession number P17301 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 85; Inverted forming-2 (INF2) that is downregulated in patients with an adverse outcome. In preferred embodiments INF2 has Uniprot accession number Q27J81 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 86; Platelet glycoprotein 4 (CD36) that is downregulated in patients with an adverse outcome. In preferred embodiments CD36 has Uniprot accession number P16671 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 87; Beta-2-glycoprotein 1 (APOH) that is downregulated in patients with an adverse outcome. In preferred embodiments APOH has Uniprot accession number P02749 (Version 3), for example having the amino acid sequence as set forth in SEQ ID NO. 88; Integrin beta-1 (ITGB1) that is downregulated in patients with an adverse outcome. In preferred embodiments ITGB1 has Uniprot accession number P05556

(Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 89; Ras GTPase-activating protein 3 (RASA3) that is downregulated in patients with an adverse outcome. In preferred embodiments RASA3 has Uniprot accession number Q14644 (Version 3), for example having the amino acid sequence as set forth in SEQ ID NO. 90; Ficolin-3 (FCN3) that is downregulated in patients with an adverse outcome. In preferred embodiments FCN3 has Uniprot accession number O75636 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 91; Platelet endothelial cell adhesion molecule (PECAM1) that is downregulated in patients with an adverse outcome. In preferred embodiments PECAM1 has Uniprot accession number P16284 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 92; ATP synthase subunit alpha, mitochondrial (ATP5A1) that is downregulated in patients with an adverse outcome. In preferred embodiments ATP5A1 has Uniprot accession number P25705 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 93; Alpha-1-antichymotrysin (SERPINA3) that is downregulated in patients with an adverse outcome. In preferred embodiments SERPINA3 has Uniprot accession number P01011 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 94; Plasma protease C1 inhibitor (SERPING1) that is downregulated in patients with an adverse outcome. In preferred embodiments SERPING1 has Uniprot accession number P05155 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 95; Protein disulfide-isomerase (P4HB) that is downregulated in patients with an adverse outcome. In preferred embodiments P4HB has Uniprot accession number P07237 (Version 3), for example having the amino acid sequence as set forth in SEQ ID NO. 96; Platelet glycoprotein Ib beta chain (GP1BB) that is downregulated in patients with an adverse outcome. In preferred embodiments GP1BB has Uniprot accession number P13224 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 97; SLC25A5 protein (SLC25A5) that is downregulated in patients with an adverse outcome. In preferred embodiments SLC25A5 has Uniprot accession number Q6NVC0 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 98; Ras-related protein Rap-1A (RAP1A) that is downregulated in patients with an adverse outcome. In preferred embodiments RAP1A has Uniprot accession number P62834 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 99; Syntaxin-binding protein 2

(STXBP2) that is downregulated in patients with an adverse outcome. In preferred embodiments STXBP2 has Uniprot accession number Q15833 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 100; Anthithrombin-III (SERPINC1) that is downregulated in patients with an adverse outcome. In preferred embodiments SERPINC1 has Uniprot accession number P01008 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 101; Calnexin (CANX) that is downregulated in patients with an adverse outcome. In preferred embodiments CANX has Uniprot accession number P27824 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 102; Solute carrier family 2 (SLC2A1) that is downregulated in patients with an adverse outcome. In preferred embodiments SLC2A1 has Uniprot accession number Q0P512 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 103; Alpha-2-antiplasmin (SERPINF2) that is downregulated in patients with an adverse outcome. In preferred embodiments SERPINF2 has Uniprot accession number P08697 (Version 3), for example having the amino acid sequence as set forth in SEQ ID NO. 104; Platelet basic protein (PPBP) that is downregulated in patients with an adverse outcome. In preferred embodiments PPBP has Uniprot accession number P02775 (Version 3), for example having the amino acid sequence as set forth in SEQ ID NO. 105; Lipopolysaccharide-binding protein (LBP) that is downregulated in patients with an adverse outcome. In preferred embodiments LBP has Uniprot accession number P18428 (Version 3), for example having the amino acid sequence as set forth in SEQ ID NO. 106; Synaptotagmin-like protein 4 (SYTL4) that is downregulated in patients with an adverse outcome. In preferred embodiments SYTL4 has Uniprot accession number Q96C24 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 107; Syntaxin-4 (STX4) that is downregulated in patients with an adverse outcome. In preferred embodiments STX4 has Uniprot accession number Q12846 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 108; Synaptotagmin-10 (STY10) that is downregulated in patients with an adverse outcome. In preferred embodiments STY10 has Uniprot accession number Q6XYQ8 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 109; Tyrosine-protein kinase (TXK) that is upregulated in patients with an adverse outcome. In preferred embodiments TKX has Uniprot accession number P42681 (Version 3), for example having the amino acid

sequence as set forth in SEQ ID NO. 110; Aldehyde dehydrogenase 1 family, member L1 variant (Fragment) (ALDH1L1) that is upregulated in patients with an adverse outcome. In preferred embodiments ALDH1L1 has Uniprot accession number Q59G10 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 111; Sodium-dependent phosphate transport protein 2C (SLC34A3) that is upregulated in patients with an adverse outcome. In preferred embodiments SLC34A3 has Uniprot accession number Q8N130 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 112; Kinetochore-associated protein 1 (KNTC1) that is upregulated in patients with an adverse outcome. In preferred embodiments KNTC1 has Uniprot accession number P50748 (Version 1), for example having the amino acid sequence as set forth in SEQ ID NO. 113; and Cytochrome c oxidase subunit 6B1 (COX6B1) that is upregulated in patients with an adverse outcome. In preferred embodiments COX6B1 has Uniprot accession number P14854 (Version 2), for example having the amino acid sequence as set forth in SEQ ID NO. 114.

[0071] The term "polypeptide" as used herein in its typical meaning refers to a polymer of amino acids and includes isolated and/or purified polypeptides, with "isolated/purified" meaning that said polypeptide has been separated from at least some other (cellular) components that may accompany it in a given sample. In various embodiments, the polypeptides may be in its natural modified form, for example, glycosylated, acetylated, phosphorylated and the like. In the context of the present invention, the term includes peptides that are 20 to about 1000 amino acids in length.

[0072] The polypeptides may also include fragments or variants of the sequences set forth in SEQ ID Nos. 1-114, comprising sequences preferably 80%, 90% or 95% identical to any one of the sequences set forth in SEQ ID NOS 1-114 over their entire length. The term "percent sequence identity" is taken to include an amino acid sequence which is at least 80%, 90% or 95% identical, preferably at least 98 or 99% identical at the amino acid level over at least 10, 20, 50, 100, 200 or 300 contiguous amino acids with the given marker amino acid sequence. Preferred polypeptides of the invention comprise a contiguous sequence having greater than 80 or 90% identity, to one or more of amino acids set forth in any one of SEQ ID Nos. 1 to 114 within each alternative sequence.

[0073] "Fragments", as used herein, relates to N- or C-terminally shortened

derivatives of the polypeptides. Preferably, the fragments are still long enough to be specifically detectable by detection reagents specific for the full length marker. This may mean that they are long enough to comprise amino acid sequences that are characteristic for a given marker. In preferred embodiments, the fragments are at least 20, preferably at least 30 amino acids in length. In other preferred embodiments, the fragments have at least 70, preferably at least 80, more preferably at least 90% of the length of the full length polypeptide.

[0074] In various embodiments the at least one biomarker is selected from the group consisting of polypeptides having the amino acid sequence set forth in SEQ ID Nos. 4, 6, 8, 13, 18, 21, 25, 31, 44-47, and 52-74 and an increased level is indicative for (i) an unfavorable prognosis, (ii) that said subject has suffered ischemic stroke or (iii) has an increased likelihood to suffer ischemic stroke.

[0075] In such embodiments, the at least one biomarker may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or 20 of the biomarkers identified above.

[0076] In various embodiments the biomarker is selected from the group consisting of polypeptides having the amino acid sequence set forth in SEQ ID NOS 12, 19, 27, 28, 48-51, 75-109 and a decreased level is indicative for (i) an unfavorable prognosis, (ii) that said subject has suffered ischemic stroke or (iii) has an increased likelihood to suffer ischemic stroke.

[0077] Again, in such embodiments, the at least one biomarker may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 of the biomarkers identified above.

[0078] In various embodiments, the biomarker is selected from the group consisting of polypeptides having the amino acid sequence set forth in SEQ ID Nos. 31, 44-47 and an increased level is indicative for (i) an unfavorable prognosis, (ii) that said subject has suffered ischemic stroke or (iii) has an increased likelihood to suffer ischemic stroke.

[0079] In various embodiments the biomarker is selected from the group consisting of polypeptides having the amino acid sequence set forth in SEQ ID NOS 28, 48-51 and a decreased level is indicative for (i) an unfavorable prognosis, (ii) that said subject has suffered ischemic stroke or (iii) has an increased likelihood to suffer ischemic stroke.

[0080] In various embodiments, the method using the markers listed above is a method for determining the prognosis of a subject afflicted by lacunar infarction.

[0081] A further aspect of the invention relates to a panel of biomarkers for determining the prognosis of a subject afflicted by ischemic stroke, predicting the risk of a subject developing ischemic stroke or diagnosing ischemic stroke in a subject comprising at least one, preferably at least two, more preferably at least 5 biomarkers selected from the group defined above in connection with the described methods.

[0082] The term "panel" as used herein may refer to at least one biomarker, or a plurality of biomarkers such as at least two biomarkers, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 12, at least 14, at least 15, or at least 20 biomarkers the presence or level of which are measured in a sample, and used for determining a diagnosis or prognosis related to ischemic stroke, or for differentiating between a non-adverse outcome and an adverse outcome after a subject has been afflicted by ischemic stroke. Such a panel may be analyzed in a number of fashions well known to those of skill in. the art. For example, each member of a panel may be compared to a "normal" value, or a value indicating a particular outcome. A particular diagnosis/prognosis may depend upon the comparison of each marker to this value; alternatively, if only a subset of biomarkers is outside of a normal range, this subset may be indicative of a particular diagnosis/prognosis.

[0083] In various embodiments each biomarker on the panel may be represented by a suitable detection reagent, whereby the biomarker is detected using the suitable detection reagent. The type of said reagent is dependent on the type of biomarker to be detected. In case the biomarker is a protein, polypeptide or peptide, the detection reagent may, for example, be an antibody or antibody-like molecule or known derivatives thereof. In various embodiments, the detection reagents may also include conjugates of the respective binding moeities, as already defined above.

[0084] In various embodiments the biomarker is selected from the group consisting of polypeptides having the amino acid sequence set forth in SEQ ID NOS 4, 6, 8, 13, 18, 21, 25, 31, 44-47, 52-74 and an increased level is indicative for (i) an unfavorable prognosis, (ii) that said subject has suffered ischemic stroke or (iii) has an increased likelihood to suffer ischemic stroke.

[0085] In various embodiments the biomarker is selected from the group consisting of polypeptides having the amino acid sequence set forth in SEQ ID NOS 12, 19, 27, 28, 48-51, 75-109 and a decreased level is indicative for (i) an unfavorable prognosis, (ii) that said subject has suffered ischemic stroke or (iii) has an increased

likelihood to suffer ischemic stroke.

[0086] In various embodiments the biomarker is selected from the group consisting of polypeptides having the amino acid sequence set forth in SEQ ID NOS 31, 44-47 and an increased level is indicative for (i) an unfavorable prognosis, (ii) that said subject has suffered ischemic stroke or (iii) has an increased likelihood to suffer ischemic stroke.

[0087] In all above embodiments, the at least one biomarker may comprise 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 of the biomarkers identified above.

[0088] In various embodiments, the biomarkers comprise the polypeptides having the amino acid sequences set forth in SEQ ID Nos. 31, 44-47.

[0089] In various embodiments, the biomarkers comprise the polypeptides having the amino acid sequences set forth in SEQ ID Nos. 31, 44 and 46.

[0090] In various embodiments, the biomarker is selected from the group consisting of polypeptides having the amino acid sequence set forth in SEQ ID NOS 28, 48-51 and a decreased level is indicative for (i) an unfavorable prognosis, (ii) that said subject has suffered ischemic stroke or (iii) has an increased likelihood to suffer ischemic stroke.

[0091] In various embodiments the at least biomarkers comprise polypeptides having the amino acid sequences set forth in SEQ ID Nos. 28, 48-51.

[0092] A further aspect of the invention relates to use of the panel of biomarkers disclosed herein for determining the prognosis of a subject afflicted by ischemic stroke, predicting the risk of a subject developing ischemic stroke or diagnosing ischemic stroke in a subject.

[0093] Also encompassed is a method for determining the prognosis of a subject afflicted by ischemic stroke, predicting the risk of a subject developing ischemic stroke or diagnosing ischemic stroke in a subject by use of the biomarker panel described above.

[0094] It should be understood that all embodiments disclosed above in relation to the methods or uses of the invention, are similarly applicable to each method and use and vice versa.

[0095] As already described above, the therapeutic importance of plasma microvesicles and the technical advantage of the quantitative method holds great promise for understanding the etiology, pathophysiology, and more importantly,

prognosis and diagnosis of subjects afflicted by lacunar infarction. The invention described herein results from establishing a proteomic imprint from plasma microvesicle samples of LACI patients. Interestingly, it has been found that the up-regulation of several platelet related proteins including the proteins of integrin pathway and coagulation cascade, probably due to a failure of an anti-platelet therapy, is associated with adverse outcome in the LACI patients. Reverse regulation of MBP and ALB could indicate underlying pathological changes ongoing during the convalescent stage of LACI.

[0096] As the plasma levels of many of the biomarkers identified herein are modifiable via drugs or changes in lifestyle, the perturbed biomarkers alone or 'as a panel' can be tested to stratify the high-risk group of patients on priority for hospital admission, treatment or rehabilitation or to monitor the effect of therapy on the long-term functional outcome of the disease. Conversely, these proteins can also be used as surrogate markers in LACI related clinical trials to monitor the consequences of therapeutic interventions. The described biomarkers thus also facilitate a better understanding of the underlying pathology of LACI affected brain samples.

[0097] Some significantly perturbed microvesicle proteins in the patients with adverse outcomes, including recurrent vascular events and cognitive decline, are identified and listed in Figures 5, 6 and 7. These plasma microvesicle biomarkers as originated from damaged brain tissue are generally applicable for diagnosis and prognosis of stroke, particularly for asymptomatic/silent stroke, LACI and transient ischemic attack (TIA) stroke subtypes.

[0098] By "comprising" it is meant including, but not limited to, whatever follows the word "comprising". Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present.

[0099] By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present.

[00100] The inventions illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the

terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention 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 inventions embodied therein 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.

[00101] The invention has been described broadly and generically herein. Each of the narrower species and sub-generic groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[00102] Other embodiments are within the following claims and non- limiting examples.

EXAMPLES

[00103] Examples 1-18 detail the identification of biomarkers that can be used to, diagnose a patient that has had an ischemic cerebral small-vessel disease (SVD), including lacunar infarction (LACI), asymptomatic/silent stroke, and transient ischemic attack (TIA) stroke subtypes or to prognose the likelihood of adverse effects after a subject has suffered a lacunar infarction (LACI).

[00104] Examples 19 and 20 detail optimization of the method of isolating microvesicles from plasma.

[00105] Examples 21-24 detail the validation of a panel of the identified biomarkers to, diagnose a patient that has had a ischemic cerebral small-vessel disease (SVD), including lacunar infarction (LACI), asymptomatic/silent stroke, and transient ischemic attack (TIA) stroke subtypes or to prognose the likelihood of adverse effects after a subject has suffered a lacunar infarction (LACI).

Reagents

[00106] Unless indicated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO).

Example 1: Sample Collection

[00107] Plasma was collected from forty five patients following a non-disabling LACI along with seventeen age/vascular risk factor-matched control subjects. The LACI patients were monitored prospectively for up to five years for the occurrence adverse outcomes (i.e. recurrent vascular events or cognitive decline). Microvesicles isolated from the pooled plasma by sequential centrifugation of four groups (control, n=17; LACI-no adverse outcome, n=19; LACI-recurrent vascular event, n=11; and LACI-cognitive decline without any recurrence of vascular events, n= 15) were profiled by an iTRAQ-2D-LC-MS/MS approach to identify the differential proteomic signature. The plasma samples were obtained from patients with a non-disabling ischemic stroke who were recruited at the Singapore General Hospital between 1999 and 2005 for the cognitive sub-study of the European Australasian Stroke Prevention in Reversible Ischemia Trial (ESPRIT-cog). Detailed methodology of ESPRIT and ESPRIT-cog including the exclusion criteria has been reported previously. Briefly, for ESPRIT, patients were eligible if they were within 6 months of a transient ischemic attack (including transient monocular blindness) or a non-disabling ischemic stroke (grade ≤3 on the modified Rankin scale [mRS]) (Bonita, R.; Beaglehole, R., *Stroke* **1988,** *19*, 1497–1500) of presumed arterial origin. Stroke subtype was classified according to the Oxfordshire Community Stroke Project (OCSP) as total anterior circulation infarct (TACI), partial anterior circulation infarct (PACI), posterior circulation infarct (POCI), or LACI. All patients were randomized to either aspirin (100 mg/day) or aspirin combined with dipyridamole (75-450 mg/day). The control plasma was collected from non-stroke subjects at the same site during 2004-2006. EDTA was used as the anti-coagulant during the processing of blood samples.

Example 2: Standard Protocol Approvals, Registrations, and Patient Consents

[00108] The study protocol was approved by Singapore General Hospital's and

Nanyang Technological University's Institutional Review Board and Ethics Committee. Written informed consent was obtained from all patients or legal guardians. The ESPRIT trial was registered under http://clinicaltrials.gov with the identifier NCT00161070.

Example 3: Neuropsychological Test Battery – Determination of Cognitive Impairment

[00109] The cognitive status of the patients was determined by trained research psychologists using standard neuropsychological test battery that has been validated for use in Singapore. Details of the procedure has been described previously (Narasimhalu, K.; Ang, S.; De Silva, D. A.; Wong, M. C.; Chang, H. M.; Chia, K. S.; Auchus, A. P.; Chen, C. P., The prognostic effects of poststroke cognitive impairment no dementia and domain-specific cognitive impairments in nondisabled ischemic stroke patients. *Stroke* **2011,** *42* (4), 883-8) incorporated herein by reference. Briefly, the battery assessed 6 domains; 2 memory domains (i.e. Verbal Memory and Visual memory) and 4 non-memory domains (Attention, Language, Visuomotor speed and Visuoconstruction). Failure in at least half of the tests in a domain constituted failure in that domain. Diagnoses of dementia were made according to the DSM-IV criteria.[19] The patients who did not qualify to be demented, included individuals with diagnoses of cognitive impairment no dementia (CIND) –mild (impairment of 1-2 domains), CIND –moderate (impairment of 3-6 domains) and no cognitive impairment (NCI).

Example 4: Experimental Design guided by Outcome Measures

[00110] This proteomic study is an analysis of 45 selected plasma samples out of 289 LACI patients, enrolled in the ESPRIT cohort at the Singapore site. The experimental design is described in Figure 1. The cognitive status of the LACI patients was determined at the baseline (3-4 months after the qualifying event) using standard neuropsychological test battery. Accordingly, the LACI patients were classified in any of the four groups namely, NCI, CIND-mild, CIND-moderate and dementia. Any patients who were demented at the baseline were excluded from this study. Next, the patients were followed up yearly up to a maximum of 5 yrs to document their cognitive status. The LACI Patients whose cognitive status declined

from the respective baseline status during the course of the prospective study had been assigned in the "cognitive decline" group. The LACI patients were also monitored for the occurrence of any vascular event annually for up to 5 years. Strokes, peripheral artery disease, intracranial bleeds, and any cardiac ischemia (stable and unstable angina, myocardial infarctions) or deaths from any of the above were considered to be a recurrent vascular event. Any LACI patient having a recurrence of vascular event during the follow-up period was included in the group called "recurrent vascular event". Patients who did not suffer a recurrence or cognitive decline during this period were named as "LACI, no adverse outcome". Accordingly, plasma samples of 45 LACI patients were divided into three groups based on the outcome variables (LACI- no adverse outcome, n=19; LACI- recurrent vascular events, n=11; LACI – cognitive decline but no recurrent vascular events, n=15). The age-matched control group had 17 subjects who never had a stroke or cancer and were cognitively normal at the baseline.

[00111] The plasma samples were pooled group-wise before processing. Microvesicles were isolated by sequential centrifugation and were labeled with isobaric tags followed by 'two dimensional liquid chromatography' to improve the depth of identification and quantification. The iTRAQ samples were injected thrice in the MS machine (technical replicate = 3).

[00112] Blood-based biomarker studies in the area of ischemic stroke mostly correlated acute levels of biomarkers (within first week after stroke onset) to short term outcome (e.g. death, disability or infarct volume) without focusing on certain subtypes of ischemic stroke. Most of the investigational biomarkers are proteins of extra-cranial source that are related to inflammation, cardiovascular system and hemostasis apart from few proteins of brain origin. The study, being a profiling approach, has identified differentially regulated peripheral as well as brain-specific candidates targeting only lacunar stroke in a single experiment, while relating them to long-term outcome measures such as cognitive decline and recurrent vascular events. In addition, the plasma was collected during the convalescent phase following the index event to evade the acute systemic response that may confound data interpretation.

Example 5: Separation of Plasma Microvesicles by Sequential Centrifugation

[00113] Frozen individual plasma samples were thawed on ice and pooled in a group-wise manner to obtain four tubes containing around 5 ml of plasma specimens from each group. The samples were subjected to sequential centrifugation to isolate the microvesicles using a modified protocol as described previously (Grant, R.; et al. *Journal of Immunological Methods* **2011,** *371* (1-2), 143-51). Briefly, sonicated plasma (5 X 1 min) was centrifuged at 4000g twice for 30 min and then at 12 000g for 30 min to collect and remove the pellets for separate studies. The resulting supernatant was subsequently diluted approx. five times with ice-cold 1X PBS before doing ultra-centrifugation at 30 000g for 2 h to collect the pellet of plasma membrane derived vesicles. The supernatant was ultra-centrifuged again at 200 000g for 2 h 15 min to collect the microvesicle pellet (Figure 2). The microvesicle pellets were washed at least twice with 1X PBS and were lyophilized. The lyophilisate was dissolved using 50-100 µl of ice-cold dissolution buffer [6% sodium dodecyl sulfate; 20 mM dithiothreitol (DTT), 100 mM tris-HCl with Complete Protease Inhibitor Cocktail (COMPLETE, (Roche; Mannheim, Germany)), pH 7.75] by brief vortexing. Protein quantization was performed using 2-D Quant kit (Amersham Biosciences, Piscataway, NJ).

Example 6: In-Gel Tryptic Digestion and Isobaric Labeling

[00114] The samples (500 μg / condition) were subjected to denaturing PAGE using a 4% - 6% - 25% gel following an identical procedure as described previously in Datta et al.. Briefly, after overnight in-gel digestion and extraction of the peptides, the dried peptides were reconstituted into 0.5 M triethylammonium bicarbonate (TEAB) and labeled with respective isobaric tags of 4-plex iTRAQ Reagent Multi-Plex kit (Applied Biosystems, Foster City, CA) as follows: Control, 114; LACI-no adverse outcome, 115; LACI- recurrent vascular events, 116; LACI – cognitive decline but no recurrent vascular events, 117 (Figure 1). Two parallel iTRAQ experiments were run one each for plasma membrane derived vesicles and exosomes.

Example 7: Electrostatic Repulsion and Hydrophilic Interaction Chromatography (ERLIC)

[00115] The combined iTRAQ sample was desalted by Sep-Pak C18 SPE cartridges (Waters, Milford, MA). A modified ERLIC using volatile salt-containing buffers was adopted. The dried iTRAQ-labeled peptide was reconstituted in 200 µl of Buffer A (10 mM NH_4HCO_2 , 85% ACN, 0.1% formic acid (FA)) and fractionated using a PolyWAX LP column (200 × 4.6 mm; 5 µm; 300 Å) (PolyLC, Columbia, MD) on a Prominence HPLC system (Shimadzu, Kyoto, Japan) in a 65 min gradient with Buffer B (30% ACN, 0.1% FA). The HPLC gradient was composed of 100% buffer A for 10 min; 0-25% buffer B for 35 min; then 25-100% buffer B for 10 min; followed by 100% buffer B for 10 min. The chromatogram was recorded at 214 nm. Eluted fractions were collected in every 1 min, and then pooled into 34 fractions depending on the peak intensities, before drying them in a vacuum centrifuge. They were stored at -20 °C till MS analysis. Most of the MS parameters were also kept similar except minor modifications. The maximum accumulation time was set at 1.0 s instead of 2.0 s that was used previously (Datta, A.; et al. *Journal of Proteome Research* **2011,** *10* (11), 5199-213) incorporated herein by reference.

Example 8: Reverse Phase LC-MS/MS Analysis using QSTAR

[00116] The iTRAQ-labeled peptides were reconstituted with 0.1% FA, 3% ACN and analyzed using a HPLC system (Shimadzu) coupled with QSTAR Elite Hybrid MS (Applied Biosystems/MDS-SCIEX) as described previously with minor modifications. Briefly, most of the LC parameters for a 90 min gradient including column configuration, gradient and flow rate were kept constant except the mobile phase A composition (0.1% FA in 3% ACN) and sample injection volume (15 µl/injection). Regarding MS parameters, the precursors with a mass range of 300–1600 m/z and calculated charge of +1 to +5 were selected for the fragmentation. The selected precursor ion was dynamically excluded for 20 s with a 50 mDa mass tolerance. All other parameters were kept identical as reported previously. The peak areas of the iTRAQ reporter ions reflect the relative abundance of the proteins in the samples.

Example 9: Mass Spectrometric Raw Data and Bioinformatics Analysis

[00117] The Analyst QS 2.0 software (Applied Biosystems) was used for the spectral data acquisition. ProteinPilot Software 3.0, Revision Number: 114 732 (Applied Biosystems) was used for the peak list generation, protein identification and quantification against the concatenated target-decoy Uniprot human database (total 191242 proteins). Details of the analysis strategy have been described previously (Datta et al. 2011).

Example 10: Bioinformatics Analysis

[00118] The bioinformatics analysis was performed using different attributes such as gene ontology (GO), pathway, protein interaction, tissue specificity, keywords or protein domains of DAVID to extract out hidden trends and enrichment of certain groups of proteins. DAVID uses modular enrichment analysis where the term–term/gene–gene relationships are considered for enrichment *p*-value calculation. It calculates the probability of the number of genes in the list that hit a given biology class as compared to pure random chance with the aid of Fisher's exact test (Huang da, W.; et al. *Nucleic Acids Research* **2009,** *37* (1), 1-13). The enriched list of annotations only facilitates a rational exploration as final selection is purely based on a priori biological knowledge of the pathology of interest. It is not uncommon to ignore specific group of proteins even with an extremely good *p*-value.

[00119] Data mining revealed (1) significant up-regulation of a group of proteins (e.g. MBP and GFAP) from brain tissue in the plasma microvesicles and proteins related to the coagulation cascade (e.g. FGA, FGB) and focal adhesion (e.g. ITGA2B, TLN1, and FLNA); (2) significant down-regulation of ALB, in the plasma microvesicles of groups of patients with adverse outcome in comparison with patients with no adverse outcome, when age-matched control group was used as a common background. This panel of candidates could be useful as surrogate markers in LACI related clinical trials.

Example 11: Western Blot (WB) Analyses

[00120] WB was performed after SDS-PAGE by probing with primary antibodies

at the indicated dilutions: anti-ALB (albumin, 1:5000, rabbit polyclonal; Abcam, Cambridge, UK) and anti-MBP (MBP, 1:1000, goat polyclonal; Santa Cruz Biotechnology, CA., USA). 40μg of proteins were used for WB. The membranes were incubated with the respective secondary antibodies from Sigma at 1:5000 dilutions (anti-rabbit HRP and anti-goat HRP). Immunoreactivity was detected by using an HRP chemiluminescent substrate reagent kit (Invitrogen, Carlsbad, CA).

[00121] MBP was detected to be significantly increased in individuals having recurrent vascular events compared to controls (Figure 12).

Example 12: Patient demography

[00122] The demographic characteristics of the study population are summarized in Figure 2. Overall, the groups were not significantly different in terms of baseline cognitive classification or demographic characteristics.

Example 13: Proteomics

[00123] The plasma microvesicles were isolated in order to enrich the low abundant proteins during the bottom-up quantitative profiling. The microvesicles provided a reservoir of potential biomarkers.

[00124] The discovery of circulatory biomarkers for neurological disorders represents an additional challenge as brain parenchyma remains selectively accessible by the systemic circulation due to the presence of blood brain barrier (BBB) under the physiological condition. This makes blood an indirect reflector to sense any events happening inside the brain tissue. However, different cell types in the brain (e.g. microglia and oligodendrocytes) are reported to release microvesicles for delivering signals to the neighboring cells and external environment. A fraction of these microvesicles may drain into the cerebrospinal fluid (CSF) or eventually in the blood. In addition, ischemic small vessel disease is well-known to cause an endothelial dysfunction and a diffuse increase in the BBB permeability that may facilitate the leakage of microvesicles in the general circulation. Hence, profiling of circulatory microvesicles by quantitative proteomics during the post-stroke recovery phase may provide a technically and conceptually preferred strategy to investigate the on-going neuro-pathological processes and to discover useful prognostic markers.

Example 14: Quality Control and Filtering of iTRAQ Data Set

[00125] To minimize the false-positive identification of proteins, a strict cutoff of unused prot-score >3 was used as the qualification criteria, which resulted in the identification of 183 proteins with a FDR of 1.1 %. Around 97% of the identified proteins had ≥2 unique peptides having a confidence of >95%. Decreasing the unused prot-score cut-off to widely used >2.0 although increases the number of identified proteins (288 proteins); makes the FDR unacceptably high (>11%). Similar observation is however been reported in a study dealing with shotgun proteomics of plasma samples (Tu, C.; et al. *Journal of Proteome Research* **2010,** *9* (10), 4982-4991). Hence, the result reported here are either comparable (Mathivanan, S.; et al. *Journal of Proteomics* **2010,** *73* (10), 1907-20) or even better (Yadav, A. K.; et al. *PLoS ONE* **2011,** *6* (9), art. no. e24442) than similar published reports in the recent past. Moreover, all these studies addressed technical issues only and none of them tried to quantify the proteome. The current approach not only provided quantitative information of these proteins but also used a simpler protocol to achieve this.

[00126] Next, a cut-off of *p*-value <0.05 was used for filtering the proteins with significant ratios from each condition. There were 17, 33 and 28 proteins for the three ratios (i.e. 115/114, 116/114 and 117/114) respectively with an acceptable *p*-value after excluding the keratins from the list. Based on the statistical evaluation of the Proteinpilot software, these values are considered as real and not due to random technical variations. Of note, this *p*-value is not related to the biological variation as a pooling strategy was adopted during the proteomic sample preparation. The groups with adverse outcome (either recurrent vascular event or cognitive decline) following a single LACI event had higher percentage of perturbed proteins in the plasma microvesicles in comparison with the LACI patients with a good recovery profile. Overall, 43 proteins having at least one ratio with an acceptable level of confidence were shortlisted for bioinformatics analysis to retrieve useful biological trends (Figure 4). Taken together, the significantly perturbed microvesicle proteins in the patients with adverse outcomes, including recurrent vascular events and cognitive decline, are listed in Figures 5,6 and 7. These plasma microvesicle biomarkers as originated from damaged brain tissue are generally applicable for diagnosis and prognosis of stroke, particularly for asymptomatic/silent stroke, LACI and transient ischemic attack (TIA)

stroke subtypes.

Example 15: Bioinformatics Analysis of perturbed plasma proteome

[00127] Uniprot accession numbers of the filtered (i.e. 43 proteins) list of proteins was uploaded in DAVID to compare them with the human proteome which was used as the background. To check the enrichment, *p*-value ≤0.01 and FDR <1% was used as cut-off. Initially, the enrichment in terms of tissue specificity was checked using the 'UP_TISSUE' module. The 43 proteins were assigned to nine different tissues that include plasma, liver, platelet, serum. As expected, 'plasma' secured the top slot with 23 proteins. The GO analysis indicated complementary trends as 'extracellular region or space' and 'platelet alpha granule' were listed as the most significantly enriched 'cellular component'. Searching for enriched pathways according to KEGG or BIOCARTA showed 'complement and coagulation cascades' and 'focal adhesion' as significantly over-represented. GO analysis in the 'biological process' category shortlisted 'acute inflammatory response', 'lipid transport' and 'response to wounding' as some of the perturbed processes whereas 'enzyme (peptidase and endopeptidase) inhibitor activity' was the key 'molecular function' that was enriched in the LACI patients.

[00128] The proteins related to complement and coagulation cascade exhibited a differential and mixed trend between groups with adverse and no adverse outcome. Proteins like FGB were down-regulated, while PLG, C5 and C3 were up-regulated in LACI patients with no adverse outcome. An opposite trend was seen for all of them in the groups with adverse outcome. In contrast, the lipoproteins did not show a differential regulation between groups. Most of the lipoproteins (APOE, APOA1, APOA2 and APOL1) were down-regulated except LPA which was significantly up-regulated across the LACI groups in comparison with the control. Despite that, the magnitude of deregulation was generally higher (e.g. LPA, APOA1) in groups with adverse outcome when compared with the group with no adverse outcome. Proteins related to 'enzyme inhibitor activity' (e.g. C5, C3, PROS1 and ITIH4) were generally up-regulated in LACI group with better outcome and down-regulated in LACI groups with adverse outcome. Interestingly, proteins related to 'focal adhesion' (e.g. ITGA2B, TLN1, FLNA) were down-regulated in LACI patients with no adverse

outcome while up-regulated in groups with adverse outcome. Overall, the trends of regulation of the significantly perturbed proteins in both groups with adverse outcome (recurrent vascular events and cognitive decline) were similar in most cases (except FGG, C4BPA) amid differences in magnitudes only. Intriguingly, the extent of deregulation is generally more for the 'recurrent vascular event' group compared to the 'cognitive decline' group. This could indicate the involvement of vascular abnormality in both groups which may remain at a subclinical stage in the patients with cognitive decline.

Example 16: Up-regulation of Integrin Signaling – Failure of Aspirin Therapy

[00129] Down-regulation of some candidates from the coagulation cascade (e.g. FGB) and integrin signaling pathway (e.g. ITGA2B, FLNA and TLN1) and up-regulation of PLG in the LACI patients was associated with no adverse outcome. PLG is secreted as a zymogen and activated by proteolysis through tissue plasminogen activator to generate plasmin, which dissolves fibrin in blood clots and helps to restore circulation. ITGA2B or Integrin alpha-IIb/beta-3 ($\alpha_{IIb}\beta_{III}$) or CD41 is a platelet membrane glycoprotein and receptor for diverse ligands including fibronectin, fibrinogen, PLG, prothrombin, and thrombospondin. Activated ITGA2B mediates platelet spreading and aggregation on vascular surfaces during hemostasis and thrombosis. It has been shown that TLN1 can independently activate β integrin by binding on its cytoplasmic tail (Ye, F.; et al. *Journal of Cell Biology* **2010,** *188* (1), 157-73). FLNA on the other hand can compete with TLN1 for binding to integrins, thereby regulating its activation under certain circumstances (Kiema, T.; et al. *Molecular Cell* **2006,** *21* (3), 337-47). In a recent study, involvement of TLN1-dependent activation of ITGA2B or Rac1 in platelets has been demonstrated for late phase stability of thrombus on undisrupted endothelial cells (Nishimura, S.; et al. *Blood* **2011,** *doi:10.1182/blood-2011-09-381400*). Thus, the overall suppression of integrin signaling in patients with no adverse outcome is complementary to the down-regulation of its ligands (FGA or FGB) and up-regulation of PLG. Aspirin has been reported to partially inhibit the inside-out ITGA2B signaling apart from its anti-platelet action (Bhatt, D. L.; Topol, E. J., *Nature Review Drug Discovery* **2003,** *2* (1), 15-28.). Considering all LACI patients were on aspirin therapy, down-regulation of

the pro-aggregatory platelet proteins and suppression of proteins from integrin signaling pathway in platelet should be related to the desired anti-thrombotic effect. This speculation was further validated as all the above-mentioned proteins (i.e. ITGA2B, TLN1, FLNA, FGA, FGB, and PLG) showed opposite or no regulation in both groups with adverse outcome (Figure 8A). High plasma fibrinogen is well-studied to be an independent risk factor for stroke and is associated with an increased risk of recurrent cardiovascular events, when stroke sub-types were not specified (Resch, K. L.; Ernst, E.; Matrai, A.; Paulsen, H. F., Fibrinogen and viscosity as risk factors for subsequent cardiovascular events in stroke survivors. *Ann. Intern. Med.* **1992,** *117* (5), 371-5). In another study dealing with small vessel disease in particular, a positive correlation was obtained between fibrinogen level and the amount of leukoaraiosis (Marti-Fabregas, J.; et al. *Eur. Neurol.* **2002,** *48* (4), 185-90). Fibrinogen is one of the main determinants of plasma viscosity. Thus, higher levels of fibrinogen in surviving LACI patients may aggravate the cerebrovascular dysfunction through hemorheologic impairment or by inducing a state of hypercoagulability.

Example 17: Up-regulation of Brain-specific MBP – predictor of poor outcome

[00130] The results clearly identified MBP with five unique peptides (unused score = 10.7) by a proteomic profiling approach justifying the utility of this tool for sensitive detection of low abundant plasma proteins. The result indicates that significantly higher MBP concentration during convalescent stage is associated with adverse outcome which is consistent with previous reports. BBB abnormality is generally more diffuse in small vessel stroke compared to non-lacunar stroke subtypes that may cause gradual and sustained leakage of brain-specific MBP into general circulation. Hence, the release of MBP, which is a structural component of CNS myelin and a marker of oligodendrocyte, either signifies an increased glial injury or an increased permeability of the BBB. Both of these may be responsible for the recurrent vascular event or cognitive decline in the groups with adverse outcome. Leaked MBP along with other CNS specific proteins may also act as antigenic signals to activate systemic immune response that could exacerbate the ischemic injury through inflammatory pathways.

Example 18: Down-regulation of ALB – indicative of poor outcome

[00131] The results show that significant down-regulation of plasma ALB is associated with adverse outcome among the surviving LACI patients Figure 8B. Several clinical studies have reported higher concentration of circulatory ALB at admission which is predictive of a better functional outcome and lower mortality in ischemic stroke patients (Idicula, T. T.; et al. *Cerebrovasc. Dis.* **2009,** *28* (1), 13-7.). However, for the first time, a complementary trend is observed by focusing on patients with non-disabling lacunar stroke only. ALB is known to be neuroprotective in preclinical animal models of stroke and is under clinical trial as a potential neuroprotective agent (Belayev, L.; et al. *Stroke* **2001,** *32* (2), 553-60). It might play a beneficial role through its antioxidant, prothrombolytic action or by promoting and sustaining perfusion in the cerebral microcirculation (Nimmagadda, A.; et al. *Stroke* **2008,** *39* (1), 198-204).48 Hence, a procoagulatory condition as discussed previously is complementary with the down-regulation of ALB in the groups with poor outcome.

[00132] A panel of plasma microvesicles' proteins such as Integrin-linked protein kinase, and other proteins targeting different aspects of the pathology (e.g. endothelium dysfunction and blood brain barrier abnormality) of lacunar infarction (LACI) are differentially present in patients' plasma compared to the healthy population. These aberrant regulations are associated with either the recurrence of vascular events or decline in cognitive functions.

Example 19: Adapted method of isolating microvesicles from plasma

[00133] Noting that 5ml of plasma from LACI patients and controls were initially used to identify the biomarker by iTRAQ quantitative proteomics. A method was developed to use less than 500μl of human plasma for the detection of microvesicle biomarkers from individual patients. This quantity may easily be obtained for an exploratory blood test in LACI patients. An ultracentrifugation protocol was adapted first using a sucrose density gradient to isolate plasma microvesicles.

[00134] Several Biomarkers were detected in Orbitrap Elite whereby around 15 times fold of increase in MBP was identified by label free MS/MS in individual LACI patients with recurrent vascular events compared to controls using 500μl of plasma (Figure 13A).

[00135] MBP differences in microvesicles fraction from 500μl were also validated by Western blot. The Western blot results show the high detection sensitivity after the use of the ultracentrifugation protocol (Figure 13B)

Example 20: Adapted method for quickly isolating microvesicles from plasma

[00136] Noting that the above established ultracentrifugation protocol requires multiple complex steps, is long (2 days) and difficult to perform, a simpler protocol was developed to improve the isolation of microvesicles using smaller plasma volume (between 100μl. and 500μl.). This is expected to increase the biomarker detection sensitivity for a successful identification with a detection kit. The affinity of the microvesicle membrane proteins for hydrophobic microenvironments was taken into account during the development of the new protocol. The method used "soluble protein precipitation for microvesicle purification method" to selectively precipitate and remove soluble proteins in the samples, leaving behind the soluble membrane enclosed microvesicles in the supernatant. Thus microvesicles can be separated from the soluble proteins in the samples. As an example, organic solvents such as acetone, acetonitrile and methanol can be used for soluble protein precipitation for microvesicles isolation.

[00137] During the application of the new protocol the plasma samples are initially mixed with organic solvent (acetone, acetonitrile and methanol) to procure the precipitation of soluble proteins and the formation of protein pellet. A spin at 15000rpm is next performed to the mix. Once the pellet has been structured the supernatant is collected. The supernatant fraction containing the microvesicles can be used for further study of the isolated microvesicles.

[00138] The new protocol shows high isolation capacity from only 100 μl of plasma to detect and discriminate the MBP presence in LACI patients that exhibit recurrent vascular events (Figure 14).

[00139] Changes on the MBP biomarker intensity between individual LACI subjects has been clearly established in these experiments. MBP can be considered a good biomarker for prediction of recurrent stroke and cognitive decline in LACI subjects.

[00140] Improvements on the microvesicles isolation protocol for sensitive

identification using smaller amount of plasma (100μl) has been established. The protocol can be performed in about 1 hour. This ensures the good performance of a kit for detection of MBP and other biomarkers in plasma microvesicles of LACI subjects.

Example 21: Selection of biomarkers to diagnose LACI and predict outcome after LACI

[00141] A panel of 20 selected candidates including GFAP (SEQ ID NO. 46), CKB (SEQ ID NO. 60), PPBP (SEQ ID NO. 105), SYTL4 (SEQ ID NO. 107), APOM (SEQ ID NO. 74), DPYL2 (SEQ ID NO. 45), APP (SEQ ID NO. 115), MBP (SEQ ID NO. 31), PIF (SEQ ID NO. 47), TPI1 (SEQ ID NO. 61), FTH1 (SEQ ID NO. 57), TXK (SEQ ID NO. 110), ALDH1L1 (SEQ ID NO. 111), NPT2C (SEQ ID NO. 112), KNTC1 (SEQ ID NO. 113), CX6B1 (SEQ ID NO. 114), ITA2B (SEQ ID NO. 28), STX4 (SEQ ID NO. 108), RASA3 (SEQ ID NO. 90), and BCHE (SEQ ID NO. 44), has been selected for a dual validation strategy of Multiplex Luminex Technology and mass spectrometric multiple reaction monitoring-based targeted proteomic approach in a large (n=200) prospectively studied cohort of LACI and control (healthy and large vessel disease) patients.

Example 22: Sample collection

[00142] The plasma samples for the validation study came from the same cohort of LACI patients and controls described in Example 1 above, but include different patients. The LACI patients were grouped based on different outcomes (i.e., recurrent vascular events but no cognitive decline, incident cognitive decline and no adverse outcome) in the similar fashion as done previously (Figure 2). The samples have been collected for a prospective study along with the baseline risk factor profile, cognitive status and outcome details following the protocol as mentioned previously (Figure 2).

[00143] Figure 11 describes the final phase involving larger groups (n= 20-100/group, patients) of LACI patients and age-matched healthy (n= 250) and LDV (n= 35) controls (Phase II in Figure 11). Although, the number of patients in the sub-groups based on outcome following LACI is variable; the total number of LACI patients is 160.

[00144] Most of the contemporary biomarker studies for stroke suffer from a common problem in the experimental design due to the lack of a mimicking control

(control samples having pathophysiological or etiologic similarity with the target disorder).The resulting biomarkers although sensitive and specific with respect to healthy control fails subsequently especially on the scale of specificity when symptomatically similar patients having a different disorder are included. To address this, a group of LDV patients (n= 15 + 35) are included at two phases of the validation study to improve the specificity of the selected biomarkers.

Example 23: Validation of Candidate Biomarkers

23.1 Microvesicles (Exosome and PMV (plasma membrane derived vesicle)) Isolation

[00145] Already optimized differential centrifugation based microvesicles' isolation strategy was used or an alternative centrifugation-free protocol for exosome isolation only using the commercial reagent Exoquick (System Biosciences). Alternatively, the isolation techniques described in examples 19 and 20 above were used.

[00146] For the ultracentrifugation method the crude plasma was sequentially centrifuged (initially at 4000g) and subsequently with the supernatants from previous centrifugation at different speeds (12 000g, 30 000g and 200 000g) to collect the pellets. This differential centrifugation helps to reduce the sample complexity and provides moderately pure PMV and exosomes at 30 000g and 200 000g respectively. The PMV and exosomes will be used for subsequent analysis. Alternatively, the commercial reagent Exoquick (System Biosciences) will be used for specifically isolating the exosomes by starting with as little as 100-250 µl of plasma following the manufacturer's protocol. This is a one-step precipitation protocol to enrich the exosomes from plasma. The choice will depend on the available plasma amount and compatibility with the downstream validation techniques.

23.2 Luminex Assay

[00147] The protein extraction by using commercial 'complete lysis M buffer' (Roche has been found to be compatible with the Luminex assay with reproducible results. Multiplex Luminex Technology is built on proven, existing technology.

Featuring a flexible, open-architecture design, Luminex technology can be configured to perform a wide variety of bioassays quickly, cost-effectively and accurately. First, Luminex color-codes tiny beads, called microspheres, into 100 distinct sets. Each bead set can be coated with a reagent specific to a particular bioassay, allowing the capture and detection of specific analytes from a sample. Within the Luminex compact analyzer, lasers excite the internal dyes that identify each microsphere particle, and also any reporter dye captured during the assay. Many readings are made on each bead set, further validating the results. In this way, this technology allows multiplexing of up to 100 unique assays within a single sample, both rapidly and precisely. This combines the power of flow cytometry with the simplicity of an ELISA plate reader. The multiplex Luminex assay format differs from conventional ELISA in one significant way: the multiplex capture antibody is attached to a polystyrene bead whereas the ELISA capture antibody is attached to the microplate well.

[00148] A Luminex bead assay that uses two antibodies and a recombinant protein was established. A Luminex assay for a marker protein is considered suitable for quantitative protein analysis in this (research) phase of the project when the criterion of good linear response of the calibrators is met.

23.3 Quantitative enzyme-linked immunosorbent assay (ELISA)

[00149] ELISA kits are vastly used in clinical diagnostic and clinical research for their reliability and simplicity of use. Sandwich ELISA displays high sensitivity and excellent specificity for the detection and quantitation of a specific protein from a small amount of sample. ELISA kits containing specific antibodies against the following biomarkers (myelin basic protein, MBP; butirylcholinesterase, BCHE; dermicidin, PIF; glial fibrillary acidic protein, GFAP; dihydropirimidinase-related protein 2, DPYSL2, and others) have been made for use in quantifying the biomarker level in plasma microvesicle.

[00150] The specific detection antibody against the biomarker antigen is pre-coated into the microplate, the standards provided and our subject samples are pipetted into the wells and any biomarker present in the sample is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated

antibody specific for the biomarker is added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added to the wells. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of biomarker bound to the specific detection antibody in the initial step. The color development is stopped and the intensity of the color is measured. The minimum amount of biomarker detectable by ELISA kits is typically less than 0.05 ng/ml. The intra and inter-assay precision of typical kits is in a range between 8-10% of CV%.

[00151] One of the principal advantages of the use of the ELISA kits is the possibility to rapidly validate the presence and the respective amount of each biomarker from the exosomal fraction in a big number of subjects (almost 90 each kit), including sample from all the predicted subjects in a short period of time.

23.4 Mass Spectrometric (MRM) Quantization for Validation of Candidate Biomarkers

[00152] When no antibody combinations plus recombinant proteins are available for the Luminex assay, targeted proteomics with MRM technology is adopted using a triple quadrupole (QqQ-MS) mass spectrometer (Agilent Technologies Inc.) as an alternative approach.

[00153] This mass spectrometry-based method is capable of multiplex quantification of proteins in a single experiment. This MRM approach monitors and quantifies the specific peptides of candidate proteins in a complex mixture. Absolute quantization (AQUA) of specific proteins in a complex mixture is achievable by internal addition of stable isotope labeled synthetic peptides.

[00154] Many candidate biomarkers do not have commercial antibody or recombinant proteins available. MRM assay is an antibody-free approach, so applicable to novel proteins for which generating an antibody in-house may be cumbersome, time-consuming and costly. The added advantage of MRM assays are their improved sensitivity and specificity, the speed at which the assays can be developed and the quantitative nature of the assay. Thus, the chances of success will become higher due to the complementary nature of both approaches and their multiplicity.

23.4 Proteomics Sample Preparation

[00155] The quantization in MRM technology happens at the level of peptides. Thus, the upstream sample handling (i.e., extraction or digestion protocol) is identical and standard operating procedure will be followed. The lyses of the microvesicles is done in 40 - 100 µl of lyses buffer (6% SDS, 20 mM DTT, protease inhibitor cocktail (complete), 100 mM Tris-Cl, pH 7.75). Protein concentration is measured by 2-D Quant kit.

[00156] For each candidate biomarker protein, four unique peptides are chosen for synthesizing stable isotope labeled heavy peptides for absolute quantization.

Example 24: Identification of Biomarkers in individual subjects

[00157] Less than 500µl of plasma from control and LACI individuals were used to isolate the microvesicles for biomarker detection based on the soluble protein precipitation method described above in example 20. The microvesicle fraction of these samples was subsequently analyzed by LC-MS/MS in an Orbitrap Elite mass spectrometer coupled with a Dionex UltiMate 3000 UHPLC system. A total of 9 of the biomarkers from the list of 20 candidates have been identified in LACI patients (recurrent vascular and cognitive decline) compared to controls (Figure 15). These include GFAP (SEQ ID NO. 46), CKB (SEQ ID NO. 60), PPBP (SEQ ID NO. 105), SYTL4 (SEQ ID NO. 107), APOM (SEQ ID NO. 74), DPYL2 (SEQ ID NO. 45), APP (SEQ ID NO. 74), MBP (SEQ ID NO. 31), and PIF (SEQ ID NO. 47).

Claims

1. A method for determining the prognosis of a subject afflicted by ischemic stroke comprising:
   a) isolating plasma microvesicles from a plasma sample obtained from the subject; and
   b) determining the presence and/or amount of at least one biomarker associated with the isolated plasma microvesicles;
   c) comparing the determined presence and/or amount of said at least one biomarker with a control, wherein a change in the presence and/or amount of the at least one biomarker relative to said control is indicative for said subject having an unfavorable prognosis.

2. The method of claim 1, wherein the unfavorable prognosis is an increased likelihood for an adverse event selected from the group of developing cognitive impairment, including dementia, and/or recurrent vascular events.

3. The method of claim 1 or 2, wherein the prognosis is a long-term prognosis, preferably a prognosis for a time period of more than 12, more than 24, more than 36, more than 48 or more than 60 months.

4. A method for predicting the risk of a subject developing ischemic stroke comprising:
   a) isolating plasma microvesicles from a plasma sample obtained from the subject; and
   b) determining the presence and/or amount of at least one biomarker associated with the isolated plasma microvesicles;
   c) comparing the determined presence and/or amount of said at least one biomarker with a control, wherein a change in the presence and/or amount of the at least one biomarker relative to said control is indicative for an increased risk of said subject developing ischemic stroke.

5. A method for diagnosing ischemic stroke in a subject comprising:

a) isolating plasma microvesicles from a plasma sample obtained from the subject; and

b) determining the presence and/or amount of at least one biomarker associated with the isolated plasma microvesicles;

c) comparing the determined presence and/or amount of said at least one biomarker with a control, wherein a change in the presence and/or amount of the at least one biomarker relative to said control is indicative for said subject having suffered an ischemic stroke.

6. The method of claim 4 or 5, wherein the subject had suffered suspected or confirmed lacunar infarction.

7. The method of any one of claims 1 to 6, wherein the subject is diagnosed with or undergoing a therapy for ischemic cerebral small vessel disease (CVD).

8. The method of any one of claims 1 to 6, wherein the ischemic stroke is selected from the group of lacunar infarction (LACI), transient ischemic attack (TIA) or silent stroke.

9. The method of any one of claims 1 to 6, wherein the control subject is a subject who has not suffered ischemic stroke.

10. The method of any one of claims 1 to 9, wherein the plasma microvesicles include exosomes.

11. The method of any one of claims 1 to 10, wherein the plasma microvesicles are isolated by ultracentrifugation, sucrose density gradient centrifugation, or soluble protein precipitation.

12. The method of any one of claims 1 to 11, wherein the amount of the at least one biomarker is determined by a combined chromatography/mass spectrometry technique, preferably LC/MS/MS technique, more preferably iTRAQ (isobaric tags for relative and absolute quantitation).

13. The method of any one of claims 1 to 12, wherein the biomarkers are selected from the group consisting of polypeptides set forth in SEQ ID NOS 1-114

14. The method of claim 13, wherein the biomarker is selected from the group consisting of polypeptides set forth in SEQ ID NOS 4, 6, 8, 13, 18, 21, 25, 31, 44-47, 52-74 and an increased level is indicative for an unfavorable prognosis, that said subject has suffered ischemic stroke or has an increased likelihood to suffer ischemic stroke.

15. The method of claim 13, wherein the biomarker is selected from the group consisting of polypeptides set forth in SEQ ID NOS 12, 19, 27, 28, 48-51, 75-109 and a decreased level is indicative for an unfavorable prognosis, that said subject has suffered ischemic stroke or has an increased likelihood to suffer ischemic stroke.

16. The method of claim 13, wherein the biomarker is selected from the group consisting of polypeptides set forth in SEQ ID NOS 31, 44-47 and an increased level is indicative for an unfavorable prognosis, that said subject has suffered ischemic stroke or has an increased likelihood to suffer ischemic stroke.

17. The method of claim 13, wherein the biomarker is selected from the group consisting of polypeptides set forth in SEQ ID NOS 28, 48-51 and a decreased level is indicative for an unfavorable prognosis, that said subject has suffered ischemic stroke or has an increased likelihood to suffer ischemic stroke.

18. The method of any one of claims 14 to 17, wherein the method is a method for determining the prognosis of a subject afflicted by lacunar infarction.

19. A panel of biomarkers for determining the prognosis of a subject afflicted by ischemic stroke, predicting the risk of a subject developing ischemic stroke or diagnosing ischemic stroke in a subject comprising at least one, preferably at least two, more preferably at least 5 markers selected from the group consisting

of polypeptides set forth in SEQ ID NOS 1-114.

20. The panel of biomarkers of claim 19, wherein the biomarker is represented by a suitable detection reagent for detection of the biomarker.

21. The panel of biomarkers of claim 19 or 20, wherein the biomarker is selected from the group consisting of polypeptides set forth in SEQ ID NOS 4, 6, 8, 13, 18, 21, 25, 31, 44-47, 52-74 and an increased level is indicative for an unfavorable prognosis, that said subject has suffered ischemic stroke or has an increased likelihood to suffer ischemic stroke.

22. The panel of biomarkers of claim 19 or 20, wherein the biomarker is selected from the group consisting of polypeptides set forth in SEQ ID NOS 12, 19, 27, 28, 48-51, 75-109 and a decreased level is indicative for an unfavorable prognosis, that said subject has suffered ischemic stroke or has an increased likelihood to suffer ischemic stroke.

23. Use of the panel of biomarkers of any one of claims 19 to 22, for determining the prognosis of a subject afflicted by ischemic stroke, predicting the risk of a subject developing ischemic stroke or diagnosing ischemic stroke in a subject.

Figure 1

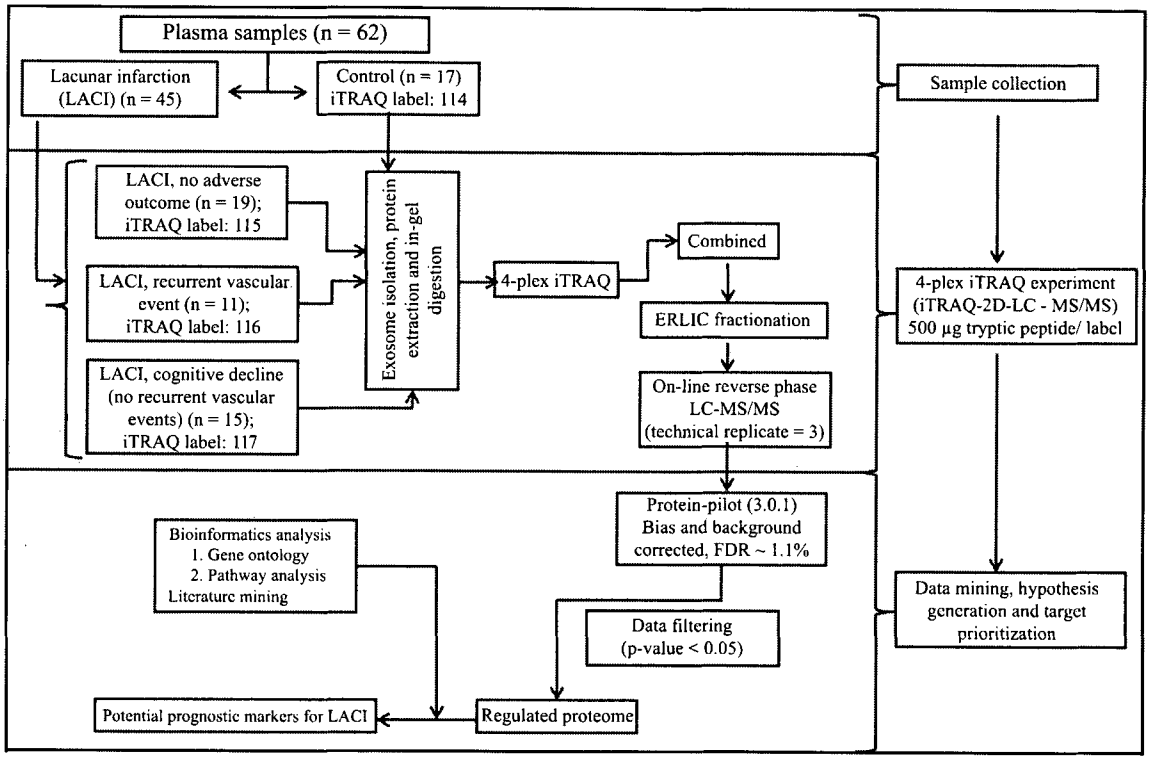

Plasma samples (n = 62)
Lacunar infarction (LACI) (n = 45)
Control (n = 17) iTRAQ label: 114
LACI, no adverse outcome (n = 19); iTRAQ label: 115
LACI, recurrent vascular event (n = 11); iTRAQ label: 116
LACI, cognitive decline (no recurrent vascular events) (n = 15); iTRAQ label: 117
Exosome isolation, protein extraction and in-gel digestion
4-plex iTRAQ
Combined
ERLIC fractionation
On-line reverse phase LC-MS/MS (technical replicate = 3)
Protein-pilot (3.0.1) Bias and background corrected, FDR ~ 1.1%
Data filtering (p-value < 0.05)
Regulated proteome
Bioinformatics analysis 1. Gene ontology 2. Pathway analysis Literature mining
Potential prognostic markers for LACI
Sample collection
4-plex iTRAQ experiment (iTRAQ-2D-LC - MS/MS) 500 µg tryptic peptide/ label
Data mining, hypothesis generation and target prioritization

Figure 2

Characteristic N (%)	No adverse outcome (N = 19)	Recurrent vascular events (stroke + MI) (N = 11)	Cognitive decline (no recurrent vascular events) (N = 15)	Healthy control (N = 17)
Age, Mean (SD)	61 (9)	65 (10)	66 (9)	56 (9)
Sex, Male	17 (90)	8 (73)	5 (33)	4 (26)
Diabetes mellitus	7 (37)	2 (18)	7 (47)	6 (35)
Hypertension	11 (58)	9 (82)	12 (80)	10 (59)
Previous stroke	0 (0)	3 (27)	4 (27)	None
Hyperlipidemia	8 (42)	4 (36)	9 (60)	10 (59)
Ever smoker	5 (26)	6 (55)	1 (7)	1 (6)
Previous ischemic heart disease	2 (11)	2 (18)	1 (7)	
Previous myocardial infarction	0 (0)	0 (0)	1 (7)	3 (18)
Previous angina	2 (11)	2 (18)	0 (0)	
Previous peripheral artery disease	0 (0)	0 (0)	0 (0)	None
Baseline cognitive classification				
NCI	13 (68)	2 (18)	8 (53)	
CIND-mild	4 (21)	6 (55)	6 (40)	None
CIND-moderate	3 (16)	3 (27)	1 (7)	

Figure 3

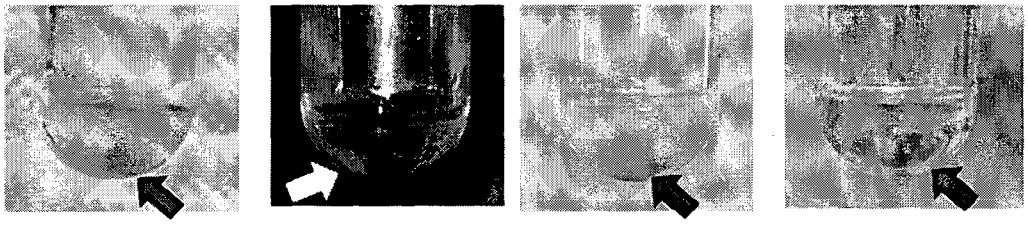


Figure 4

SEQ ID No	Unused	%Cov (95)	Accession Number	Protein name	Gene Symbol	Peptides (95%)	115:114	116:114	117:114	GO/pathway
1	1099.8	86.2	P01023	Alpha-2-macroglobulin	A2M	2474	1.00	**1.02**	**1.07**	CCC, EIA, RTW, ECS
2	257.0	68.8	P01024	Complement C3	C3	291	**1.54**	**0.22**	**0.30**	CCC, EIA, RTW, ECS, IIR
3	226.6	83.7	P02768	Serum albumin	ALB	433	1.16	**0.14**	**0.12**	ECS
4	155.1	47.9	P02671	Fibrinogen alpha chain	FGA	267	0.92	**4.79**	**3.94**	CCC, RTW, ECS
5	124.2	78.0	P02675	Fibrinogen beta chain	FGB	209	**0.29**	**2.09**	**1.20**	CCC, ECS
6	106.0	67.0	P00738	Haptoglobin	HP	171	1.94	1.80	**0.58**	ECS
7	99.4	57.2	P02679	Fibrinogen gamma chain	FGG	193	**0.30**	**1.38**	**0.55**	CCC, RTW, ECS
8	71.1	15.7	P04275	von Willebrand factor	VWF	44	**1.36**	**3.94**	**4.25**	FA, CCC, RTW, ECS
9	52.9	18.1	P01031	Complement C5	C5	29	**1.32**	**0.36**	0.56	CCC, EIA, RTW, ECS, IIR
10	51.9	8.8	Q9Y6R7	IgGFc-binding protein	FCGBP	31	1.02	**1.57**	**1.84**	ECS
11	49.1	41.7	*Q5VVQ8*	Complement component 4 binding protein, alpha	C4BPA	40	1.27	**1.42**	**0.35**	CCC, RTW, ECS, IIR
12	45.3	68.9	P02647	Apolipoprotein A-I	APOA1	30	0.77	**0.33**	**0.52**	LT, ECS
13	41.9	34.5	Q08380	Galectin-3-binding protein	LGALS3BP	44	**0.30**	**0.26**	0.78	ECS
14	41.0	14.0	*Q60FE2*	Non-muscle myosin heavy polypeptide 9	MYH9	25	0.49	**3.19**	**2.05**	
15	39.8	11.1	P21333	Filamin-A	FLNA	21	0.88	**2.94**	**2.58**	FA, ECS
16	38.6	11.0	Q9Y490	Talin-1	TLN1	19	0.65	**2.61**	**2.11**	FA, ECS
17	37.4	69.7	P69905	Hemoglobin subunit alpha	HBA1	56	**3.87**	**1.89**	0.49	
18	35.2	47.3	O43866	CD5 antigen-like	CD5L	28	**0.46**	0.86	1.04	ECS
19	32.6	38.5	P01009	Alpha-1-antitrypsin	SERPINA1	21	0.75	**0.09**	**0.20**	CCC, EIA, RTW, ECS

20	30.3	22.3	P00747	Plasminogen	PLG	14	**1.43**	0.91	1.14	CCC, RTW, ECS
21	28.5	23.8	*Q4W5C3*	Kallikrein B, plasma (Fletcher factor) 1, isoform CRA_b	KLKB1	17	**0.59**	**0.55**	1.02	CCC, RTW, ECS
22	24.9	20.3	P07225	Vitamin K-dependent protein S	PROS1	13	1.43	1.05	**0.49**	CCC, EIA, RTW, ECS
23	24.0	15.0	*B2RMS9*	Inter-alpha (Globulin) inhibitor H4 (Plasma Kallikrein-sensitive glycoprotein)	ITIH4	14	**1.69**	0.73	0.75	EIA, RTW, ECS
24	21.4	29.6	O14791	Apolipoprotein L1	APOL1	15	0.61	**0.14**	0.69	LT, ECS, IIR
25	21.3	14.7	*Q1HP67*	Lipoprotein, Lp(A)	LPA	14	**2.81**	**9.12**	**7.38**	LT, EIA, RTW, ECS
26	19.9	27.9	P02790	Hemopexin	HPX	12	1.18	0.51	**0.30**	ECS
27	16.0	27.8	P02649	Apolipoprotein E	APOE	8	0.69	**0.65**	**0.49**	LT, ECS
28	13.7	8.0	P08514	Integrin alpha-IIb	ITGA2B	6	1.12	**2.13**	1.42	FA
29	12.5	36.5	P01591	Immunoglobulin J chain	IGJ	16	0.77	**1.96**	**2.75**	ECS
30	12.2	20.6	P27169	Serum paraoxonase/arylesterase 1	PON1	6	0.75	**0.41**	**0.23**	ECS
31	10.7	21.4	P02686	Myelin basic protein	MBP	5	1.08	**14.19**	**8.87**	
32	10.2	18.3	*Q5UGI6*	Serine/cysteine proteinase inhibitor clade G member 1 splice variant 2 (Fragment)	SERPING1	5	1.10	0.70	**0.41**	CCC, EIA, ECS, IIR
33	6.4	20.0	P02652	Apolipoprotein A-II	APOA2	2	0.61	**0.48**	0.65	LT, EIA, RTW, ECS
34	76.0	42.2	*A8K5A4*	cDNA FLJ76826, highly similar to Homo sapiens ceruloplasmin (ferroxidase) (CP), mRNA		63	**1.74**	**0.13**	0.94	
35	49.5	33.1	*B2R950*	cDNA, FLJ94213, highly similar to Homo sapiens pregnancy-zone protein (PZP), mRNA		233	0.90	**0.43**	1.27	

Figure 4 Cont

36	47.1	44.5	*A8K5J8*	cDNA FLJ75066, highly similar to Homo sapiens complement component 1, r subcomponent (C1R), mRNA	31	**0.67**	**0.25**	**0.23**			
37	31.4	10.2	*B4E1Z4*	cDNA FLJ55673, highly similar to Complement factor B (EC 3.4.21.47)	13	**1.41**	**0.41**	**0.44**			
38	18.2	26.4	*Q8WVW5*	Putative uncharacterized protein (Fragment)	9	**0.09**	1.71	1.16	FA, RTW		
39	18.2	8.7	*Q59E99*	Thrombospondin 1 variant (Fragment)	8	**0.55**	1.17	1.03	FA, ECS		
40	13.4	16.1	*B3KS79*	cDNA FLJ35730 fis, clone TESTI2003131, highly similar to ALPHA-1-ANTICHYMOTRYPSIN	7	1.01	0.51	**0.38**			
41	12.2	9.6	*B7Z550*	cDNA FLJ59731, highly similar to Complement component C8 beta chain	C8B	6	0.83	**0.44**	**0.39**		
42	4.6	14.6	*A8K486*	Peptidyl-prolyl cis-trans isomerase	2	1.96	**7.05**	**5.45**			
43	3.3	2.2	*Q59GB4*	Dihydropyrimidinase-like 2 variant (Fragment)	1	1.38	**5.40**	3.84			

6/18
Figure 5

A

SEQ ID No	GeneSym	Accession	Name
31	**MBP**	**P02686**	**MBP_HUMAN Myelin basic protein OS=Homo sapiens GN=MBP PE=1 SV=3**
44	**BCHE**	**P06276**	**CHLE_HUMAN Cholinesterase OS=Homo sapiens GN=BCHE PE=1 SV=1**
45	**DPYSL2**	**Q16555**	**DPYL2_HUMAN Dihydropyrimidinase-related protein 2 OS=Homo sapiens GN=DPYSL2 PE=1 SV=1**
46	**GFAP**	**P14136**	**GFAP_HUMAN Glial fibrillary acidic protein OS=Homo sapiens GN=GFAP PE=1 SV=1**
47	**PIF**	**Q53YJ2**	**Q53YJ2_HUMAN Dermcidin OS=Homo sapiens GN=PIF PE=2 SV=1**

B

SEQ ID No	GeneSym	Accession	Name
28	**ITGA2B**	**P08514**	**ITA2B_HUMAN Integrin alpha-IIb OS=Homo sapiens GN=ITGA2B PE=1 SV=3**
48	**FERMT3**	**Q86UX7**	**URP2_HUMAN Fermitin family homolog 3 OS=Homo sapiens GN=FERMT3 PE=1 SV=1**
49	**MMRN1**	**Q13201**	**MMRN1_HUMAN Multimerin-1 OS=Homo sapiens GN=MMRN1 PE=1 SV=3**
50	**PDIA3**	**P30101**	**PDIA3_HUMAN Protein disulfide-isomerase A3 OS=Homo sapiens GN=PDIA3 PE=1 SV=4**
51	**ESYT1**	**Q9BSJ8**	**ESYT1_HUMAN Extended synaptotagmin-1 OS=Homo sapiens GN=ESYT1 PE=1 SV=1**

Figure 6

SEQ ID No	GeneSym	Accession	Name
31	MBP	P02686	MBP_HUMAN Myelin basic protein OS=Homo sapiens GN=MBP PE=1 SV=3
25	LPA	Q1HP67	Q1HP67_HUMAN Lipoprotein, Lp(A) OS=Homo sapiens GN=LPA PE=2 SV=1
47	PIF	Q53YJ2	Q53YJ2_HUMAN Dermcidin OS=Homo sapiens GN=PIF PE=2 SV=1
52	PPIA	P62937	PPIA_HUMAN Peptidyl-prolyl cis-trans isomerase A OS=Homo sapiens GN=PPIA PE=1 SV=2
53	HPR	P00739	HPTR_HUMAN Haptoglobin-related protein OS=Homo sapiens GN=HPR PE=1 SV=2
46	GFAP	P14136	GFAP_HUMAN Glial fibrillary acidic protein OS=Homo sapiens GN=GFAP PE=1 SV=1
54	HBB	C8C504	C8C504_HUMAN Beta-globin OS=Homo sapiens GN=HBB PE=3 SV=1
44	BCHE	P06276	CHLE_HUMAN Cholinesterase OS=Homo sapiens GN=BCHE PE=1 SV=1
8	VWF	P04275	VWF_HUMAN von Willebrand factor OS=Homo sapiens GN=VWF PE=1 SV=3
6	HP	P00738	HPT_HUMAN Haptoglobin OS=Homo sapiens GN=HP PE=1 SV=1
13	LGALS3BP	Q08380	LG3BP_HUMAN Galectin-3-binding protein OS=Homo sapiens GN=LGALS3BP PE=1 SV=1
4	FGA	P02671	FIBA_HUMAN Fibrinogen alpha chain OS=Homo sapiens GN=FGA PE=1 SV=2
55	HBD	D1MGP8	D1MGP8_HUMAN Delta globin (Fragment) OS=Homo sapiens GN=HBD PE=3 SV=1
18	CD5L	O43866	CD5L_HUMAN CD5 antigen-like OS=Homo sapiens GN=CD5L PE=1 SV=1
45	DPYSL2	Q16555	DPYL2_HUMAN Dihydropyrimidinase-related protein 2 OS=Homo sapiens GN=DPYSL2 PE=1 SV=1
56	PRR4	Q16378	PROL4_HUMAN Proline-rich protein 4 OS=Homo sapiens GN=PRR4 PE=1 SV=2
57	FTH1	Q6NZ44	Q6NZ44_HUMAN Ferritin OS=Homo sapiens GN=FTH1 PE=2 SV=1
58	APOL1	A5PL32	A5PL32_HUMAN APOL1 protein (Fragment) OS=Homo sapiens GN=APOL1

			PE=2 SV=1
59	PRSS1	Q3SY19	Q3SY19_HUMAN PRSS1 protein OS=Homo sapiens GN=PRSS1 PE=2 SV=1
60	CKB	P12277	KCRB_HUMAN Creatine kinase B-type OS=Homo sapiens GN=CKB PE=1 SV=1
61	TPI1	P60174	TPIS_HUMAN Triosephosphate isomerase OS=Homo sapiens GN=TPI1 PE=1 SV=2
62	PZP	A6ND27	A6ND27_HUMAN Putative uncharacterized protein PZP OS=Homo sapiens GN=PZP PE=4 SV=2
63	CRYAB	P02511	CRYAB_HUMAN Alpha-crystallin B chain OS=Homo sapiens GN=CRYAB PE=1 SV=2
64	UQCRH	P07919	QCR6_HUMAN Cytochrome b-c1 complex subunit 6, mitochondrial OS=Homo sapiens GN=UQCRH PE=1 SV=2
65	CAMK2A	Q8IWE0	Q8IWE0_HUMAN Calcium/calmodulin-dependent protein kinase II alpha OS=Homo sapiens GN=CAMK2A PE=2 SV=1
66	FTL	Q6IBT7	Q6IBT7_HUMAN Ferritin OS=Homo sapiens GN=FTL PE=2 SV=1
67	COMP	P49747	COMP_HUMAN Cartilage oligomeric matrix protein OS=Homo sapiens GN=COMP PE=1 SV=2
68		Q9NSD0	Q9NSD0_HUMAN Protein S OS=Homo sapiens PE=2 SV=1
69	CP	P00450	CERU_HUMAN Ceruloplasmin OS=Homo sapiens GN=CP PE=1 SV=1
70	RELN	C9J2G2	C9J2G2_HUMAN Putative uncharacterized protein RELN (Fragment) OS=Homo sapiens GN=RELN PE=4 SV=1
71	COX6A1	P12074	CX6A1_HUMAN Cytochrome c oxidase subunit 6A1, mitochondrial OS=Homo sapiens GN=COX6A1 PE=1 SV=4
72	COL6A3	B7ZW00	B7ZW00_HUMAN COL6A3 protein OS=Homo sapiens GN=COL6A3 PE=2 SV=1
73	APP	B2R5G8	B2R5G8_HUMAN Amyloid protein A OS=Homo sapiens PE=2 SV=1
74	APOM	Q5SRP5	Q5SRP5_HUMAN Apolipoprotein M OS=Homo sapiens GN=APOM PE=4 SV=1
21	KLKB1	Q4W5C3	Q4W5C3_HUMAN Kallikrein B, plasma (Fletcher factor) 1, isoform CRA_b OS=Homo sapiens GN=KLKB1 PE=2 SV=1

Figure 6 cont.

Figure 7

SEQ ID No	GeneSym	Accession	Name
28	ITGA2B	P08514	ITA2B_HUMAN Integrin alpha-IIb OS=Homo sapiens GN=ITGA2B PE=1 SV=3
75	VCL	P18206	VINC_HUMAN Vinculin OS=Homo sapiens GN=VCL PE=1 SV=4
48	FERMT3	Q86UX7	URP2_HUMAN Fermitin family homolog 3 OS=Homo sapiens GN=FERMT3 PE=1 SV=1
49	MMRN1	Q13201	MMRN1_HUMAN Multimerin-1 OS=Homo sapiens GN=MMRN1 PE=1 SV=3
76	ITGB3	P05106	ITB3_HUMAN Integrin beta-3 OS=Homo sapiens GN=ITGB3 PE=1 SV=2
12	APOA1	P02647	APOA1_HUMAN Apolipoprotein A-I OS=Homo sapiens GN=APOA1 PE=1 SV=1
77	GP1BA	A5CKE2	A5CKE2_HUMAN Platelet glycoprotein Ib alpha polypeptide (Fragment) OS=Homo sapiens GN=GP1BA PE=4 SV=1
50	PDIA3	P30101	PDIA3_HUMAN Protein disulfide-isomerase A3 OS=Homo sapiens GN=PDIA3 PE=1 SV=4
19	SERPINA1	P01009	A1AT_HUMAN Alpha-1-antitrypsin OS=Homo sapiens GN=SERPINA1 PE=1 SV=3
78	ATP5B	P06576	ATPB_HUMAN ATP synthase subunit beta, mitochondrial OS=Homo sapiens GN=ATP5B PE=1 SV=3
79	GP5	P40197	GPV_HUMAN Platelet glycoprotein V OS=Homo sapiens GN=GP5 PE=1 SV=1
80	ITGA6	C9JK10	C9JK10_HUMAN Putative uncharacterized protein ITGA6 OS=Homo sapiens GN=ITGA6 PE=3 SV=1
81	SLC4A1	P02730	B3AT_HUMAN Band 3 anion transport protein OS=Homo sapiens GN=SLC4A1 PE=1 SV=3
82	ILK	Q13418	ILK_HUMAN Integrin-linked protein kinase OS=Homo sapiens GN=ILK PE=1 SV=2

83	ATP2A3	Q93084	AT2A3_HUMAN Sarcoplasmic/endoplasmic reticulum calcium ATPase 3 OS=Homo sapiens GN=ATP2A3 PE=1 SV=2
84	APOA4	P06727	APOA4_HUMAN Apolipoprotein A-IV OS=Homo sapiens GN=APOA4 PE=1 SV=3
85	ITGA2	P17301	ITA2_HUMAN Integrin alpha-2 OS=Homo sapiens GN=ITGA2 PE=1 SV=1
86	INF2	Q27J81	INF2_HUMAN Inverted formin-2 OS=Homo sapiens GN=INF2 PE=1 SV=2
87	CD36	P16671	CD36_HUMAN Platelet glycoprotein 4 OS=Homo sapiens GN=CD36 PE=1 SV=2
88	APOH	P02749	APOH_HUMAN Beta-2-glycoprotein 1 OS=Homo sapiens GN=APOH PE=1 SV=3
27	APOE	P02649	APOE_HUMAN Apolipoprotein E OS=Homo sapiens GN=APOE PE=1 SV=1
89	ITGB1	P05556	ITB1_HUMAN Integrin beta-1 OS=Homo sapiens GN=ITGB1 PE=1 SV=2
90	RASA3	Q14644	RASA3_HUMAN Ras GTPase-activating protein 3 OS=Homo sapiens GN=RASA3 PE=1 SV=3
91	FCN3	O75636	FCN3_HUMAN Ficolin-3 OS=Homo sapiens GN=FCN3 PE=1 SV=2
51	ESYT1	Q9BSJ8	ESYT1_HUMAN Extended synaptotagmin-1 OS=Homo sapiens GN=ESYT1 PE=1 SV=1
92	PECAM1	P16284	PECA1_HUMAN Platelet endothelial cell adhesion molecule OS=Homo sapiens GN=PECAM1 PE=1 SV=1
93	ATP5A1	P25705	ATPA_HUMAN ATP synthase subunit alpha, mitochondrial OS=Homo sapiens GN=ATP5A1 PE=1 SV=1
94	SERPINA3	P01011	AACT_HUMAN Alpha-1-antichymotrypsin OS=Homo sapiens GN=SERPINA3 PE=1 SV=2
95	SERPING1	P05155	IC1_HUMAN Plasma protease C1 inhibitor OS=Homo sapiens GN=SERPING1 PE=1 SV=2

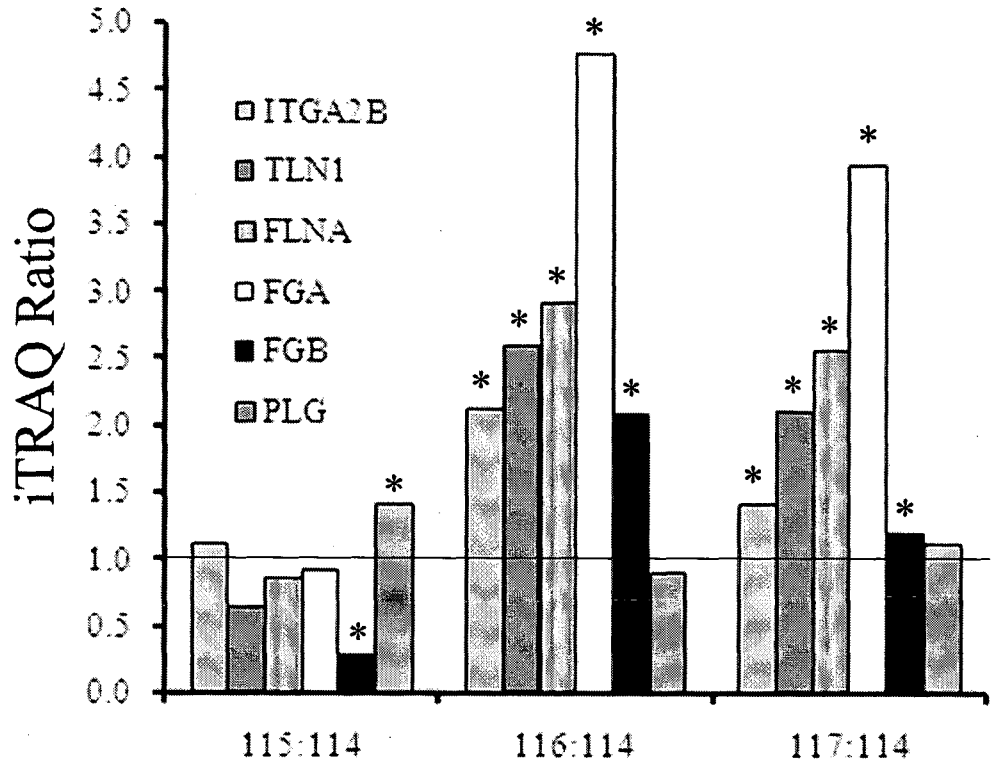
Figure 7 cont

96	P4HB	P07237	PDIA1_HUMAN Protein disulfide-isomerase OS=Homo sapiens GN=P4HB PE=1 SV=3
97	GP1BB	P13224	GP1BB_HUMAN Platelet glycoprotein Ib beta chain OS=Homo sapiens GN=GP1BB PE=1 SV=1
98	SLC25A5	Q6NVC0	Q6NVC0_HUMAN SLC25A5 protein (Fragment) OS=Homo sapiens GN=SLC25A5 PE=2 SV=1
99	RAP1A	P62834	RAP1A_HUMAN Ras-related protein Rap-1A OS=Homo sapiens GN=RAP1A PE=1 SV=1
100	STXBP2	Q15833	STXB2_HUMAN Syntaxin-binding protein 2 OS=Homo sapiens GN=STXBP2 PE=1 SV=1
101	SERPINC1	P01008	ANT3_HUMAN Antithrombin-III OS=Homo sapiens GN=SERPINC1 PE=1 SV=1
102	CANX	P27824	CALX_HUMAN Calnexin OS=Homo sapiens GN=CANX PE=1 SV=2
103	SLC2A1	Q0P512	Q0P512_HUMAN Solute carrier family 2 (Facilitated glucose transporter), member 1 OS=Homo sapiens GN=SLC2A1 PE=2 SV=1
104	SERPINF2	P08697	A2AP_HUMAN Alpha-2-antiplasmin OS=Homo sapiens GN=SERPINF2 PE=1 SV=3
105	PPBP	P02775	CXCL7_HUMAN Platelet basic protein OS=Homo sapiens GN=PPBP PE=1 SV=3
106	LBP	P18428	LBP_HUMAN Lipopolysaccharide-binding protein OS=Homo sapiens GN=LBP PE=1 SV=3
107	SYTL4	Q96C24	SYTL4_HUMAN Synaptotagmin-like protein 4 OS=Homo sapiens GN=SYTL4 PE=1 SV=1
108	STX4	Q12846	STX4_HUMAN Syntaxin-4 OS=Homo sapiens GN=STX4 PE=1 SV=2
109	SYT10	Q6XYQ8	SYT10_HUMAN Synaptotagmin-10 OS=Homo sapiens GN=SYT10 PE=2 SV=1

Figure 7 cont.

Figure 8

A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5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ITGA2B
TLN1
FLNA
FGA
FGB
PLG
115:114
116:114
117:114

B

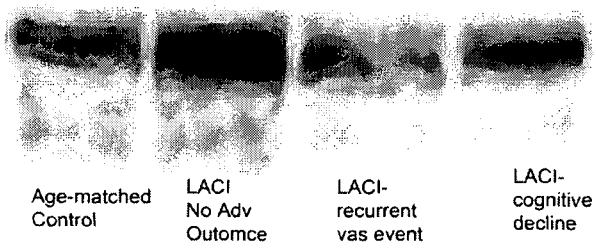
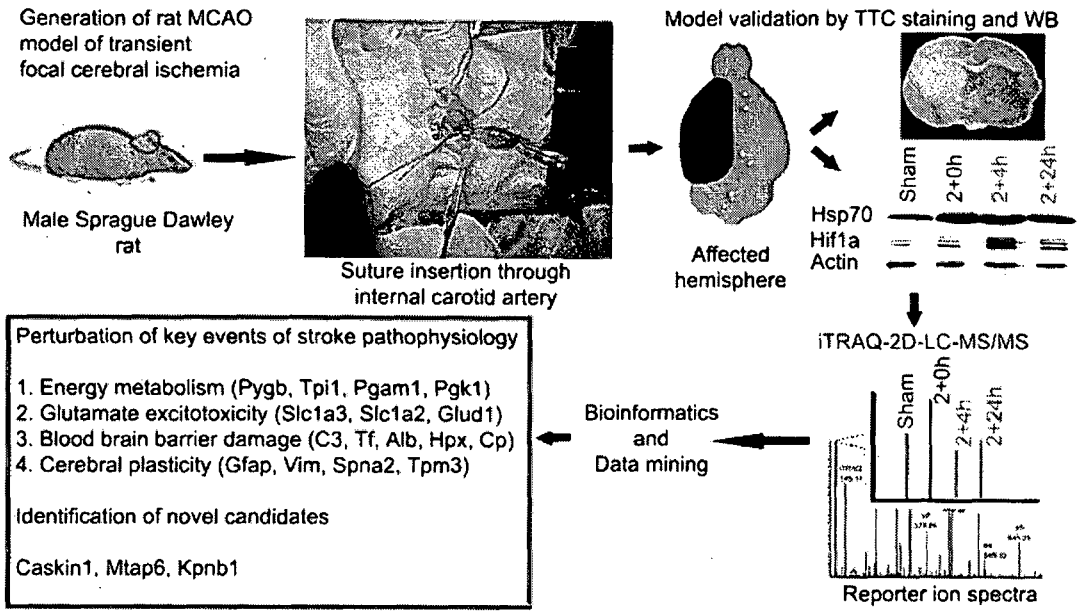
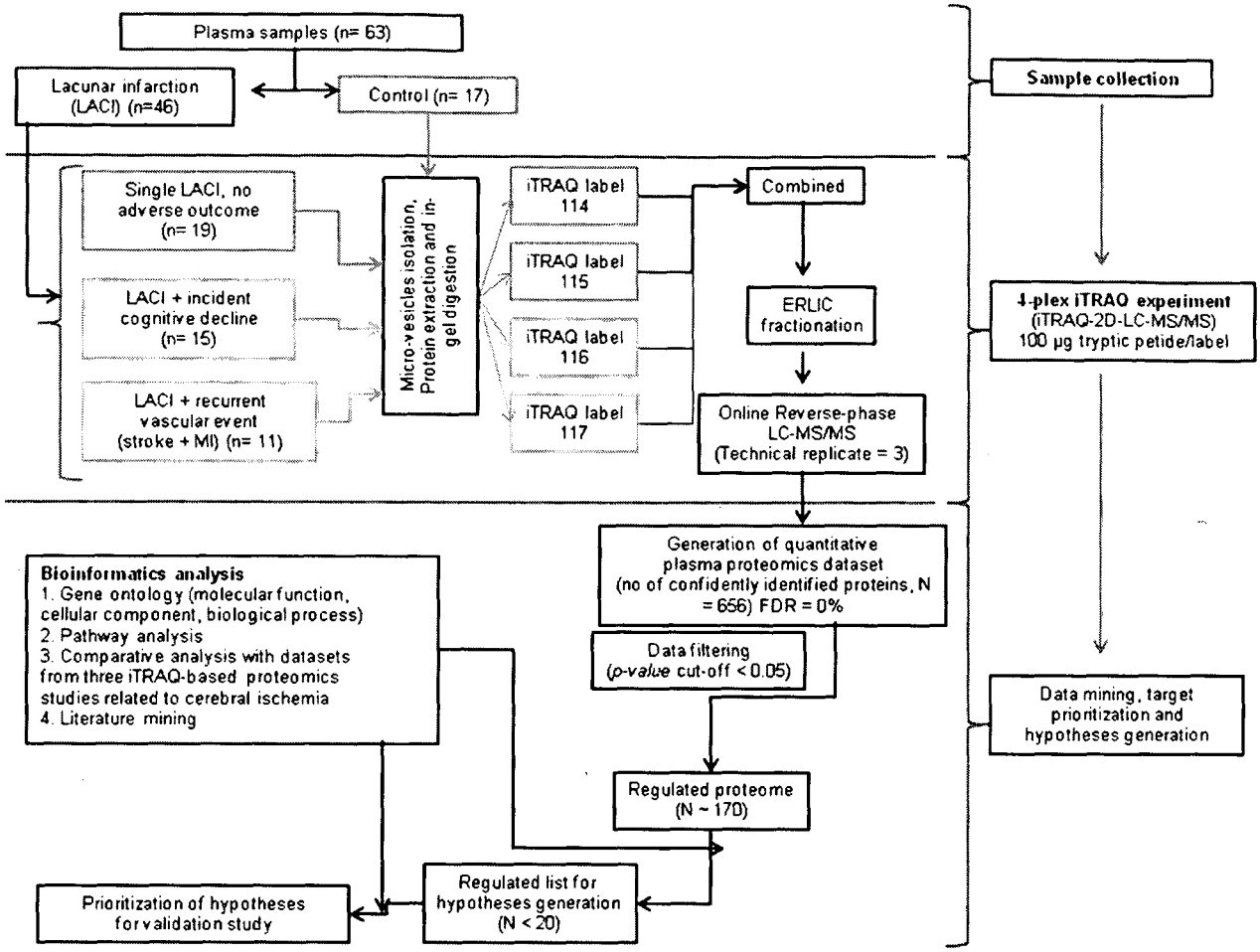

Age-matched Control
LACI No Adv Outomce
LACI-recurrent vas event
LACI-cognitive decline

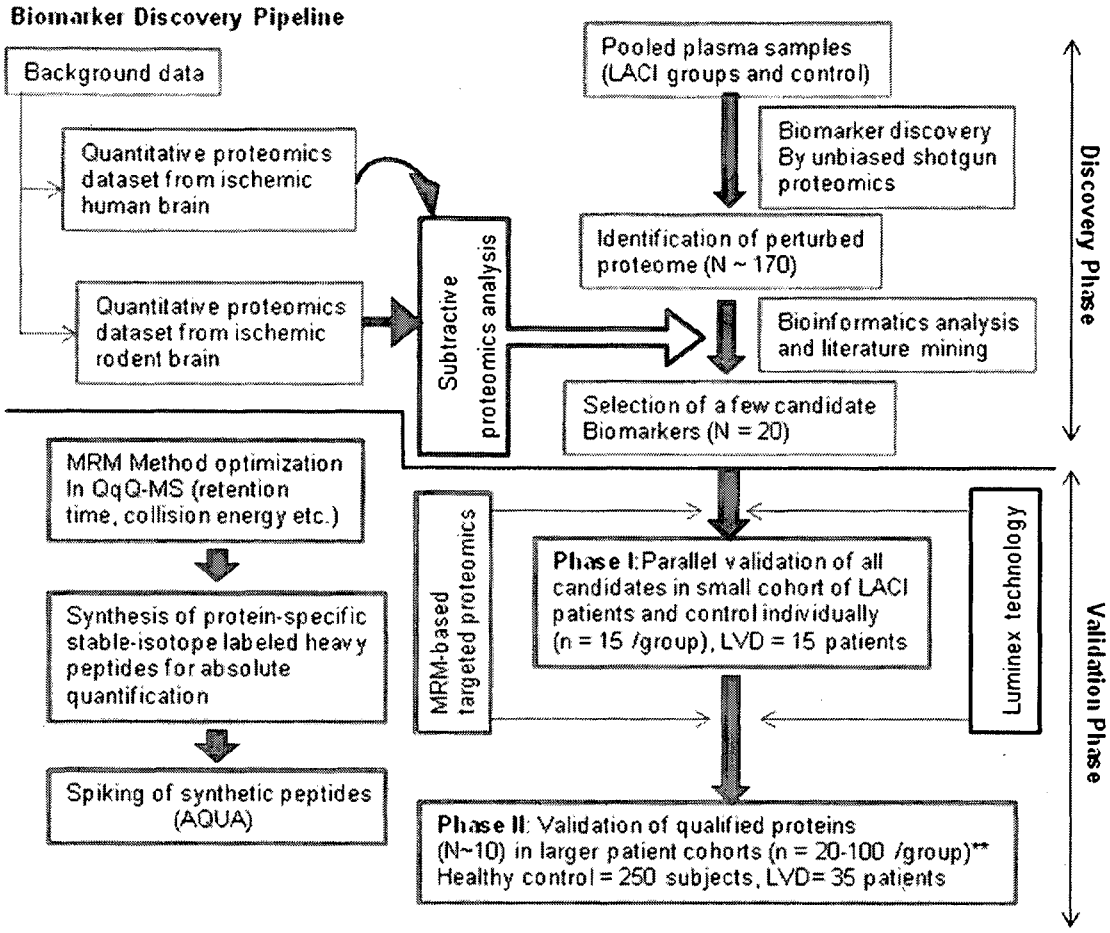
Figure 9


Generation of rat MCAO model of transient focal cerebral ischemia
Male Sprague Dawley rat
Suture insertion through internal carotid artery
Affected hemisphere
Model validation by TTC staining and WB
Sham
2+0h
2+4h
2+24h
Hsp70
Hif1a
Actin
iTRAQ-2D-LC-MS/MS
Reporter ion spectra
Bioinformatics and Data mining
Perturbation of key events of stroke pathophysiology
1. Energy metabolism (Pygb, Tpi1, Pgam1, Pgk1)
2. Glutamate excitotoxicity (Slc1a3, Slc1a2, Glud1)
3. Blood brain barrier damage (C3, Tf, Alb, Hpx, Cp)
4. Cerebral plasticity (Gfap, Vim, Spna2, Tpm3)
Identification of novel candidates
Caskin1, Mtap6, Kpnb1

14/18
Figure 10

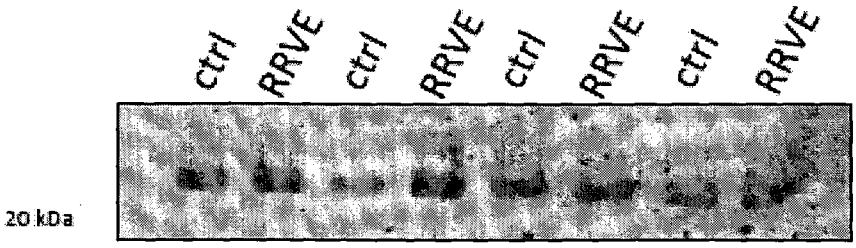


15/18
Figure 11



16/18
Figure 12

A



B

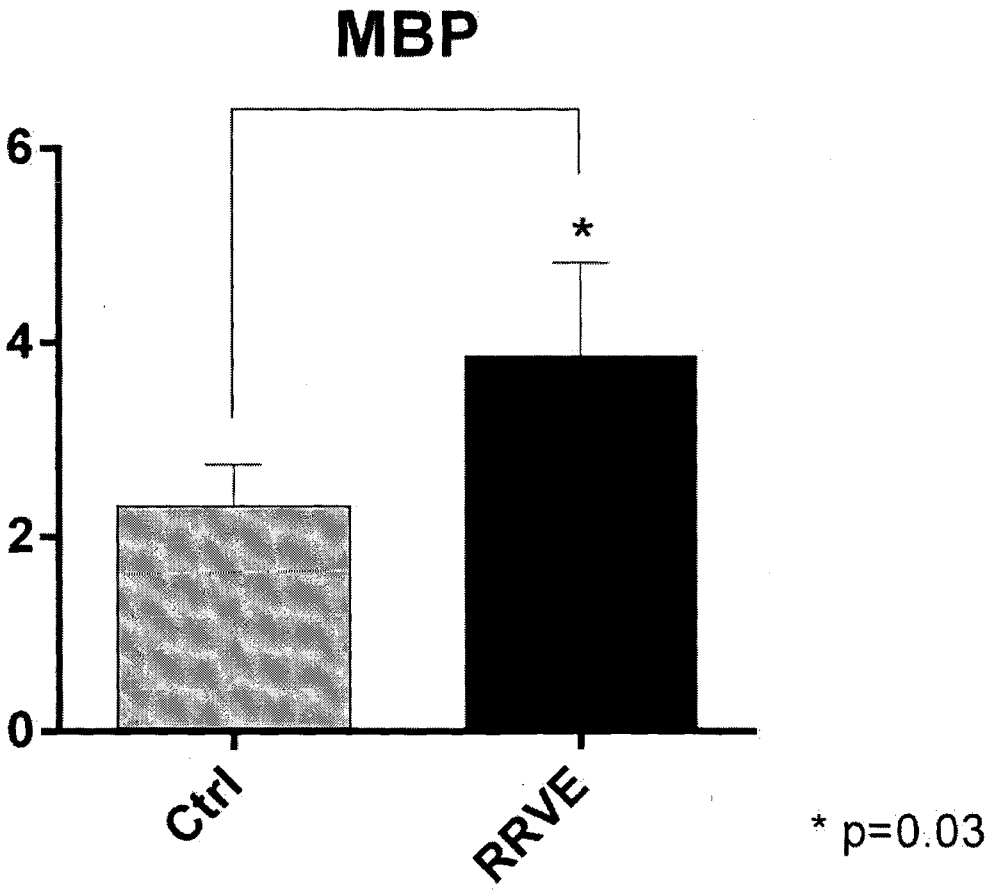


Figure 13

A

	Prot hit	Prot score	RRVE	Ctrl
J3QL64_HUMAN Myelin basic protein OS=Homo sapiens GN=MBP PE=2 SV=1	71	384	4.08	0.26
GFAP_HUMAN Glial fibrillary acidic protein OS=Homo sapiens GN=GFAP PE=1 SV=1	59	509	0.47	n.d.
F8WF14_HUMAN Cholinesterase OS=Homo sapiens GN=BCHE PE=2 SV=1	125	145	n.d.	0.11

B

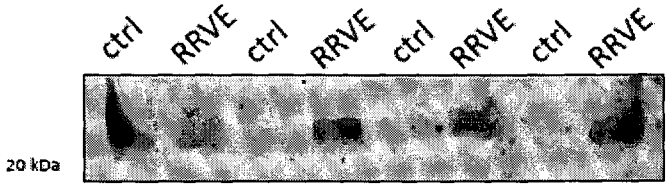


Figure 14

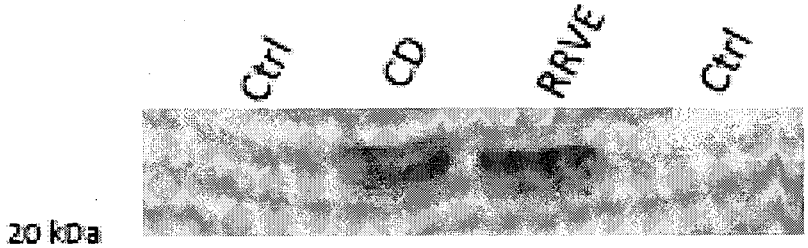
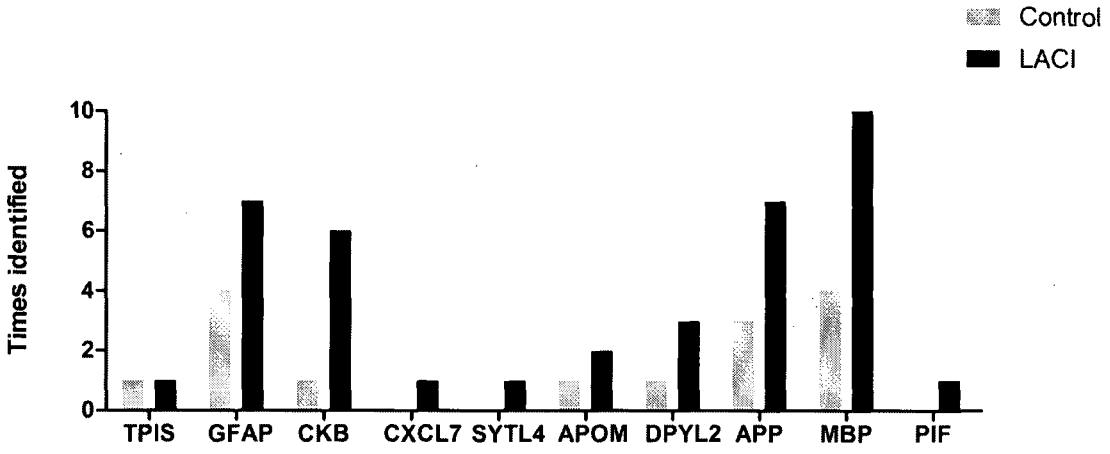


Figure 15


Control
LACI
Times identified
10
8
6
4
2
0
TPIS
GFAP
CKB
CXCL7
SYTL4
APOM
DPYL2
APP
MBP
PIF

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2014/000422

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/68 (2006.01)

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)

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

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

EPODOC, WPI, MEDLINE, HCA, BIOSIS, GENOMEQUEST, ESPACENET, PUBMED (stroke, lacunar infarction, transient ischemic attack, cerebral small vessel disease, microvesicle, plasma microparticle, exosome, biomarker, differential expression, profile, signature, SEQ ID No's 1-114 and like terms, Applicant/Inventor)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

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

[X] See patent family annex

* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 November 2014	07 November 2014
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	James Alderman AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0399359613

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☐ in the international application as filed

   ☐ together with the international application in electronic form

   ☒ subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:
   A sequence listing was furnished separate to the International application. SEQ ID No's 1-114 from this listing were used as a basis for the International Search.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SG2014/000422

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:

the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including

2. ☐ Claims Nos.:

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

3. ☐ Claims Nos:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

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

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

See Supplemental Box for Details

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.

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	**PCT/SG2014/000422**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	WO 2012/110253 A2 (CAVADIS B.V.) 23 August 2014	
X	Abstract, Claims, pages 5-8, 34-36, 47-48	1-4, 6-13, 18-23
Y	As above	5, 14-17
	WHITELEY, W. et al. "Blood Biomarkers in the Diagnosis of Ischemic Stroke: A Systematic Review", Stroke, 2008, Vol. 39, pages 2902-2909	
X	Abstract, pages 2903-2905, Figure 2	19-21, 23
	KANHAI, D.A. et al. "Microvesicle protein levels are associated with increased risk for future vascular events and mortality in patients with clinically manifest vascular disease", International Journal of Cardiology, 2013, Vol. 168 (26 Feb 2013), pages 2358-2263	
X	Abstract, Tables 1, 4, page 2362	1-4, 6-12
Y	As above	5, 13-23
	CUADRADO, E. et al. "The Proteome of Human Brain After Ischemic Stroke", Journal of Neuropathology and Experimental Neurology, 2010, Vol. 69, No. 11, pages 1105-1115	
Y	Abstract, Table	5, 13-16, 18-23
	DATTA, A. et al. "Quantitative clinical proteomic study of autopsied human infarcted brain specimens to elucidate the deregulated pathways in ischemic stroke pathology", Journal of Proteomics, 2013, Vol. 91 (2 September), pages 556-568	
Y	Abstract, Table 1	5, 13-16, 18-23
	STANKOVIC, S. & MAJKIC-SINGH, N. "Genetic aspects of ischemic stroke: coagulation, homocysteine, and lipoprotein metabolism as potential risk factors", Critical Reviews in Clinical Laboratory Sciences, 2010, Vol. 47, No. 2, pages 72-123	
Y	Abstract, Conclusions	5, 13-15, 17-23
	JICKLING, G.C. & SHARP, F.R. "Blood Biomarkers of Ischemic Stroke", Neurotherapeutics, 2011, Vol. 8, pages 349-360	
A	See whole document	
	BARON, M. et al. "Cell-derived microparticles in atherosclerosis: biomarkers and targets for pharmacological modulation?", Journal of Cellular and Molecular Medicine, 2012, Vol. 16, No. 7, pages 1365-1376	
A	See whole document	
	DATTA, A. et al. "Discovery of Prognostic Biomarker Candidates of Lacunar Infarction by Quantitative Proteomics of Microvesicles Enriched Plasma", PLoS One, 2014, Vol. 9, Issue 4:e99663	
P,X	See whole document	1-23

Supplemental Box

Continuation of: **Box III**

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

This Authority has found that there are different inventions based on the following features that separate the claims into distinct groups:

Claims 1-18 are directed towards the diagnosis/prognosis of ischemic stroke comprising the measurement of biomarkers present within plasma microvesicles. The feature of diagnosis/prognosis of ischemic stroke comprising the measurement of biomarkers present within plasma microvesicles is specific to this group of claims.

Claims 19-22 are directed towards panels of biomarkers (and uses thereof) for the diagnosis/prognosis of ischemic stroke. The feature of biomarkers panels for the diagnosis/prognosis of ischemic stroke is specific to this group of claims.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

When there is no special technical feature common to all the claimed inventions there is no unity of invention.

In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship. The only feature common to all of the claimed inventions and which provides a technical relationship among them is the use of biomarkers for the diagnosis/prognosis of ischemic stroke. However this feature does not make a contribution over the prior art because it is disclosed in documents D1-D6 as cited in herein.

In the light of these documents this common feature cannot be a special technical feature. Therefore there is no special technical feature common to all the claimed inventions and the requirements for unity of invention are consequently not satisfied *a posteriori*.

Additionally, it is noted that the claims define the use of "at least one" biomarker. Claims 13-23 define said biomarkers as relating to SEQ ID No's 1-114. As the the feature of determining the risk of suffering ischemic stroke by measuring biomarkers within microvesicles is known from prior art documents D1 and D3, it is considered appropriate to further separate the claims into individual inventions based on each individual protein defined by SEQ ID No's 1-114.

It is further noted, however, that the ISA has searched the full scope of the claims.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/SG2014/000422**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
WO 2012/110253 A2	23 August 2014	EP 2676141 A2	25 Dec 2013
		US 2014024046 A1	23 Jan 2014
		WO 2012110099 A1	23 Aug 2012
		WO 2012126531 A1	27 Sep 2012

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.