

(21) Application No: 2005632.1

(22) Date of Filing: 17.04.2020

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(51) INT CL:
G01N 33/68 (2006.01) **G01N 33/50** (2006.01)
G01N 33/53 (2006.01)

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(58) Field of Search:
INT CL **G01N**
Other: **WPI, EPODOC, Patent Fulltext, INTERNET,**
BIOSIS, MEDLINE

(54) Title of the Invention: **Method for diagnosing stroke caused by large vessel occlusion**
Abstract Title: **Biomarkers for diagnosis of stroke resulting from large vessel occlusion (LVO) and methods of diagnosis**

(57) Biomarkers for diagnosis of stroke resulting from large vessel occlusion (LVO) and methods of diagnosis
The use of two or more biomarkers to diagnose stroke resulting from large vessel occlusion (LVO), wherein the biomarkers are selected from: succinate, succinic-glutathione, N-acetyl-aspartate, propionyl-carnitine, glutamate, heart fatty acid binding protein (H-FABP), brain fatty acid binding protein (B-FABP), Abeta 1-40, OPG, soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK), pro-vWF, retinol binding protein 4 (RBP4), ADAMTS13, NMDA receptor 2 peptide (NR2 peptide), 20-HETE, bilirubin, brain natriuretic peptide (BNP), glial fibrillary acidic protein (GFAP), D-dimer, C-reactive Protein (CRP), matrix metallo- proteinase 9 (MMP9), interleukin 6 (IL-6), osteopontin (OPN), Troponin I, S100b, von Willebrand Factor (vWF) or P-selectin. Also claimed is a method of diagnosing stroke comprising determining the amount of biomarkers in a sample; a method of identifying individuals who have suffered a stroke as a result of LVO, comprising identifying the amount of at least two biomarkers in a sample; and a kit for carrying out these methods.

Method for Diagnosing Stroke caused by Large Vessel Occlusion

The invention relates to the diagnosis of stroke resulting from occlusion of one or more large vessels in the brain, and in particular to the diagnosis of stroke resulting from
5 occlusion of one or more large vessels in the brain using one or more biomarkers.

Background

Stroke affects 16 million people in the world every year. More than 30% of these patients die and 90% of survivors develop permanent disabilities. Stroke is defined by
10 the World Health Organisation as a clinical syndrome consisting of rapidly developing clinical signs of focal (or global in case of coma) disturbance of cerebral function with symptoms lasting 24 hours or longer or leading to death with no apparent cause other than a vascular origin. The definition of stroke thus excludes transient ischemic attack (TIA), which presents with the same or similar symptoms to stroke, but is a temporary
15 disruption in the blood supply to part of the brain and is defined as lasting less than 24 hours; and stroke mimics, which are non-vascular conditions which present with neurological deficits simulating stroke, which include seizures and postictal paralysis; toxic-metabolic disturbances; brain tumours; infections, and migraine.

20 There are two main types of stroke: ischemic stroke and haemorrhagic stroke. The treatment regime for each is very different due to their different aetiologies.

Haemorrhagic stroke is due to the rupture of a blood vessel in the brain with consequent bleeding. There are two main types of haemorrhagic stroke: intracerebral
25 haemorrhage (bleeding within the brain itself, due to either intraparenchymal haemorrhage (bleeding within the brain tissue) or intraventricular haemorrhage (bleeding within the brain's ventricular system)); and subarachnoid haemorrhage, (bleeding within the skull but outside of the brain tissue; more precisely, bleeding between the arachnoid mater and pia mater). Haemorrhagic stroke represents around
30 15% of stroke seen in patients.

Ischemic stroke is defined as the physical blockage of blood flow to an area of the brain, and represents around 85% of stroke seen in patients.

One type of ischemic stroke is stroke resulting from small vessel occlusion (SVO, also referred to as lacunar stroke). SVO stroke or lacunar stroke is caused by occlusion of one or more small vessels, typically that supply deep brain structures.

5 However, the deadliest type of ischemic stroke is stroke resulting from large vessel occlusion (LVO) or simply LVO stroke. LVO stroke results from occlusion of one or more large vessels in the brain¹ including the common carotid artery, basilar artery, vertebral artery, middle cerebral artery, anterior cerebral artery, posterior cerebral artery, external carotid artery, internal carotid artery and/or anterior cerebral arteries.
10 Stroke resulting from LVO contributes disproportionately to stroke-related dependence and death¹.

The typical treatment for patients identified as suffering or having suffered from LVO stroke is mechanical thrombectomy (MT). MT is a surgical procedure that
15 mechanically removes the clot via a probe inserted at the level of the groin. MT is typically only useful to treat LVO stroke, as the vessels affected in other types of ischemic strokes (such as SVO stroke) cannot be reached by the mechanical probe due to their depth within the brain and/or are too small to accommodate the mechanical probe. Treatment of LVO patients with MT significantly increases the chances of
20 survival, as well as decreases the extent of disability²⁻⁴. Mechanical thrombectomy has been proven a safe and effective treatment for LVO stroke until 24 hours from stroke onset³, indicating that detection of LVO strokes several hours after onset can significantly aid the stroke care pathway. Improved speed and/or accuracy of identification of those individuals who present with stroke symptoms who are or who
25 have suffered a LVO stroke is very important to ensure that they are treated by MT, and achieve revascularisation.

However, ischemic and haemorrhagic stroke typically present with similar symptoms. In addition, different types of ischemic stroke can present with similar symptoms.
30 Further to this, patients presenting with stroke-like symptoms may be suffering from a TIA or stroke mimic, and/or may be suffering from more than one pathology. These factors mean that accurate diagnosis of stroke type can be challenging.

Typically, diagnosis of stroke is achieved or informed using computerised tomography
35 (CT) scan to the head. CT is highly accurate for detection of brain haemorrhages, but can be very inaccurate for detection of ischemic stroke, and LVO stroke specifically. An

MRI scan may also be carried out, to aid with diagnosis of ischemic stroke and ischemic stroke type. If LVO stroke is suspected, the patient typically undergoes a further procedure, called CT angiography. Examination of CTA and/or MRI images by a neuroradiologist are then required in order to diagnose LVO stroke.

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Diagnosis of LVO stroke in an individual presenting with stroke symptoms can therefore be a lengthy procedure, and requires expert (neuroradiological) input. A delay in diagnosis and treatment negatively impacts on patient outcome. A diagnostic which is able to identify or aid identification of individuals who have suffered an LVO stroke could speed up treatment of these patients, thereby reducing death and disability rates.

Several studies have investigated the ability of clinical scores based on patient symptoms to aid in the diagnosis of LVO strokes ⁵⁻¹⁰. Such clinical scores are generally based on a simplified form of the widely applied national institute of health stroke scale (NIHSS) system, but none of them has shown improved diagnostic performance compared to the NIHSS system for the identification of LVO strokes ¹¹. Although the NIHSS system has shown promising results in the identification of LVO strokes ¹², cut-off values applied to a real clinical scenario have shown poor diagnostic performance ¹³. This indicates that clinical scores alone are not sufficient to diagnose LVO stroke.

Several documents report the use of biomarker measurements for differential diagnosis of stroke subtype. WO2012009567 describes the measurement of an expression profile to determine whether a patient is suffering an ischemic stroke. WO2016087611 describes a method for the differentiation of ischemic and haemorrhagic stroke subtypes by measurement of a panel of blood biomarkers. Other studies have assessed the ability of blood biomarkers to differentiate between stroke subtypes and aid in the acute stroke diagnosis. Sharma et al measured 262 proteins and found that a panel of 5 proteins could distinguish between any stroke type and mimics ¹⁴, but no biomarker was suggested for differentiation between stroke subtypes. Bustamante et al tested a panel of 21 biomarkers for the differentiation between stroke and mimics, as well as between ischemic stroke and haemorrhagic stroke ¹⁵. Their study concluded that the tested biomarkers were not sufficient for an accurate diagnosis of stroke, or stroke subtype, in the acute clinical setting. Other studies have attempted to identify biomarkers for the diagnosis of LVO strokes. Wang et al found a significant association between plasma soluble CD40L/P-selectin and LVO stroke patients ¹⁶. Arenillas and

colleagues observed increased amounts of blood c-reactive protein (CRP) in patients with intracranial large-artery occlusive strokes ¹⁷. Chang et al reported association between cardiac biomarkers and LVO strokes ¹⁸. However, there is currently no means for diagnosing, specifically, stroke resulting from LVO.

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Summary of the invention

In accordance with a first aspect, there is provided a method of diagnosing stroke as a result of a large vessel occlusion (LVO), comprising determining the amount of at least two biomarkers in a sample taken from an individual identified as or suspected of
10 having or having had a stroke wherein the biomarker(s) are selected from: succinate, succinic-glutathione, N-acetyl-aspartate, propionyl-carnitine, glutamate, heart fatty acid binding protein (H-FABP), brain fatty acid binding protein (B-FABP), Abeta 1-40, OPG, soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK), pro-vWF, retinol binding protein 4 (RBP4), ADAMTS13, NMDA receptor 2 peptide (NR2
15 peptide), 20-HETE, bilirubin, brain natriuretic peptide (BNP), glial fibrillary acidic protein (GFAP), D-dimer, C-reactive Protein (CRP), matrix metallo- proteinase 9 (MMP9), interleukin 6 (IL-6), osteopontin (OPN), Troponin I, s100b, von Willebrand Factor (vWF) or P-selectin.

20 In accordance with a second aspect, there is a method of identifying, from a group of individuals identified as or suspected of having or having had a stroke, individuals who have suffered a stroke as a result of LVO, comprising determining the amount of at least two biomarkers in a sample taken from each individual, wherein the biomarkers are selected from: succinate, succinic-glutathione, N-acetyl-aspartate, propionyl-
25 carnitine, glutamate, heart fatty acid binding protein (H-FABP), brain fatty acid binding protein (B-FABP), Abeta 1-40, OPG, soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK), pro-vWF, retinol binding protein 4 (RBP4), ADAMTS13, NMDA receptor 2 peptide (NR2 peptide), 20-HETE, bilirubin, brain natriuretic peptide (BNP), glial fibrillary acidic protein (GFAP), D-dimer, C-reactive
30 Protein (CRP), matrix metallo- proteinase 9 (MMP9), interleukin 6 (IL-6), osteopontin (OPN), Troponin I, s100b, von Willebrand Factor (vWF) or P-selectin.

In some embodiments methods comprise determining the amount of at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least 10,
35 at least eleven, or 12 or more biomarkers.

In some embodiments the methods comprise comparing the amount of each biomarker in the sample taken from the or each individual identified as or suspected of having or having had a stroke to the amount of the same biomarker in one or more of a control, a positive standard, one or more positive references or a negative reference.

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In some embodiments the individual is diagnosed as having or having had a stroke as a result of LVO when the amount of one or more of the measured biomarkers differs from the amount of the same biomarker in a control; or is comparable to the amount of the same biomarker in a positive standard.

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In some embodiments the methods comprise transforming the amount of one or more biomarkers measured in the sample taken from an or each individual identified as or suspected of having or having had a stroke into an LVO stroke score.

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In some embodiments, the methods comprise comparing the LVO stroke score created using the measured biomarker amounts in a sample taken from an individual identified as or suspected of having or having had a stroke to an LVO stroke score created using biomarker amounts from a control, or from a positive standard.

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In some embodiments the individual is diagnosed as having or having had a stroke as a result of LVO when the LVO stroke score created using the measured biomarker amounts in a sample taken from an individual identified as or suspected of having or having had a stroke is comparable to an LVO stroke score created using biomarker amounts from a positive standard; or when the LVO stroke score created using the

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measured biomarker amounts in a sample taken from an individual identified as or suspected of having or having had a stroke differs from the LVO stroke score created using biomarker amounts from a control.

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In some embodiments, the sample from the individual is one or more of blood, plasma, cerebrospinal fluid or saliva.

In some embodiments, the amount of biomarker is determined using an agent that binds to the biomarker. In some such embodiments the detection and/or quantification of the amount of biomarker or biomarkers comprises the use of ELISA.

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In some embodiments the method further comprises consideration of data and/or information from one or more clinical assessments which the individual has undergone. In some such embodiments the clinical assessment is one or more of a CT or CTA scan, MRA or MRI scan, or the individual's NIHSS, FAST, ABCD, ABCD2
5 Rosier, TOAST, EMSA, PASS, VAN, RACE, FAST_ED or CPSS score.

In some embodiments, the amount of biomarker in the sample taken from the individual identified as or suspected of having or having had a stroke is determined using an agent that binds to the biomarker, preferably wherein the amount of
10 biomarker is determined using ELISA.

In some embodiments, the method is carried out within about 24 hours of onset of stroke symptoms in the individual.

15 In accordance with a third aspect, there is provided the use of two or more biomarkers to detect stroke resulting from large vessel occlusion, wherein the biomarkers are selected from: succinate, succinic-glutathione, N-acetyl-aspartate, propionyl-carnitine, glutamate, heart fatty acid binding protein (H-FABP), brain fatty acid binding protein (B-FABP), Abeta 1-40, OPG, soluble tumor necrosis factor-like weak inducer of
20 apoptosis (sTWEAK), pro-vWF, retinol binding protein 4 (RBP4), ADAMTS13, NMDA receptor 2 peptide (NR2 peptide), 20-HETE, bilirubin, brain natriuretic peptide (BNP), glial fibrillary acidic protein (GFAP), D-dimer, C-reactive Protein (CRP), matrix metallo- proteinase 9 (MMP9), interleukin 6 (IL-6), osteopontin (OPN), Troponin I, s100b, von Willebrand Factor (vWF) or P-selectin.

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In some embodiments, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, at least eleven or twelve or more biomarkers are used to identify stroke resulting from large vessel occlusion.

30 In a fourth aspect there is provided a kit for carrying out a method according to the first or second aspect of the invention comprising one or more of: a container for holding a sample from the individual identified as or suspected of having or having had a stroke; a means for determining the amount of the target biomarker(s); and instructions on how to carry out the reaction between the sample and the means provided so as to
35 determine the amount of the target biomarker(s).

Detailed Description

It has been found that detection of certain amounts of specific biomarkers in a sample from an individual can determine or inform the decision as to whether the individual has suffered a stroke resulting from LVO.

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Accordingly, in a first aspect there is provided a method of diagnosing stroke as a result of a large vessel occlusion (LVO) comprising determining the amount of at least two biomarkers in a sample taken from an individual identified as or suspected of having or having had a stroke, wherein the biomarker are selected from: succinate, succinic-
10 glutathione, N-acetyl-aspartate, propionyl-carnitine, glutamate, heart fatty acid binding protein (H-FABP), brain fatty acid binding protein (B-FABP), Abeta 1-40, OPG, soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK), pro-vWF, retinol binding protein 4 (RBP4), ADAMTS13, NMDA receptor 2 peptide (NR2 peptide), 20-HETE, bilirubin, brain natriuretic peptide (BNP), glial fibrillary acidic protein (GFAP),
15 D-dimer, C-reactive Protein (CRP), matrix metallo- proteinase 9 (MMP9), interleukin 6 (IL-6), osteopontin (OPN), Troponin I, s100b, von Willebrand Factor (vWF) or P-selectin.

The phrase 'stroke as a result of large vessel occlusion (LVO)', also referred to herein as
20 'LVO stroke' means stroke resulting from occlusion of one or more of the common carotid artery, basilar artery, vertebral artery, middle cerebral artery, anterior cerebral artery, posterior cerebral artery, external carotid artery, internal carotid artery and/or anterior cerebral arteries.

25 As used herein, the phrase "an individual suspected of having or having had a stroke" refers to an individual presenting with one or more signs or symptoms associated with stroke. This can include an individual suffering from a TIA or stroke mimic. The phrase "an individual identified as having or having had a stroke" as used herein indicates an individual presenting with one or more signs or symptoms associated
30 with stroke who has, in addition, been diagnosed as having or having had a stroke.

Signs or symptoms associated with stroke include arm drift, sudden-onset face weakness, hemiplegia and muscle weakness of the face, abnormal speech as well as combination thereof such as the FAST test, reduction in sensory or vibratory sensation,
35 numbness, initial flaccidity (hypotonicity), replaced by spasticity (hypertonicity), obligatory synergies and, in particular, when they appear in one side of the body

(unilateral), altered smell, taste, hearing, or vision (total or partial), hyperreflexia, decreased reflexes (e.g. gag, swallow, pupil reactivity to light), decreased sensation and muscle weakness of the face, drooping of eyelid (ptosis) and weakness of ocular muscles, balance problems and nystagmus, aphasia, dysarthria, apraxia, altered
5 breathing and heart rate, atrial fibrillation, weakness in sternocleidomastoid muscle with inability to turn head to one side, weakness in tongue (inability to protrude and/or move from side to side), visual field defect, hemineglect, disorganized thinking, memory deficits, confusion, lack of insight of his or her, usually stroke-related, disability, altered movement coordination, altered walking gait, vertigo, hypersexual
10 gestures, headache and or disequilibrium.

In some embodiments the method comprises determining the amount of at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least
10, at least eleven, or 12 or more biomarkers.

15 The sample may be any fluid and/or tissue taken or derived from the individual identified as or suspected of having or having had a stroke. In some embodiments the sample is one or more of cells, tissues, cerebral spinal fluid (CSF), whole blood, serum, plasma, cytosolic fluid, urine, faeces, stomach fluids, tears, digestive fluids, saliva, nasal
20 or other airway fluid, vaginal fluids or semen. In some embodiments more than one sample may be taken from the individual. In some embodiments, two or more samples are taken from the individual. In some such embodiments the samples may be different, for example the samples may be whole blood and CSF; saliva and whole blood; whole blood, CSF and tears. In some embodiments, the sample from the
25 individual is one or more of blood, plasma, cerebrospinal fluid or saliva.

In some embodiments, the sample or samples taken from the individual are processed prior to analysis to detect and/or quantify any biomarker in the sample. The need for processing and/or the type of processing of the sample required depends upon the
30 biomarker to be detected and/or the assay to be used to detect the biomarker. In some embodiments, the sample may need to be diluted with, for example, saline solution to obtain a concentration appropriate for the biomarker detection assay.

A whole blood sample may be processed to provide blood plasma and/or blood serum
35 using standard procedures in the art. For example, whole blood sample may be centrifuged at 2000 g for 15 minutes at 4°C to provide blood plasma, which can then be

used directly or diluted with saline solution as required for the biomarker detection assay.

5 The sample is then analysed, to detect the presence of the target biomarker(s) and/or to determine the amount of the target biomarker(s) in the sample. Where more than one sample has been taken from the individual, analysis of the samples may be carried out simultaneously or sequentially. Samples from more than one individual may be analysed simultaneously and/or in the same assay.

10 Analysis may be carried out by any suitable means. For example, analysis may be carried out by one or more of ELISA, radioimmunoassay, radioassay, enzyme activity assay, cellular assay, western blot, Southern blot, northern blot, immunoprecipitation, immunofluorescence, liquid chromatography, high performance liquid chromatography, positron emission tomography, mass spectrometry, RT-PCR, PCR, 15 mass spectroscopy, gel electrophoresis, mass-sensing BioCD protein array, electrochemical immunoassay, surface enhanced Raman spectroscopy, fluorescence based detection, flow cytometry, nanoparticle based detection, quantum dot technology and protein microarray.

20 In some embodiments the analysis technique involves use of one or more agents that recognise and bind to a biomarker in the sample. The agent may have the ability to bind to more than one biomarker.

In some embodiments, the agent is a monoclonal or polyclonal antibody. The agent 25 may be an intact antibody, a fragment (e.g., Fab or F(ab')₂), or an engineered variant thereof (e.g., sFv). Such antibodies can be of any immunoglobulin class, and any subclass thereof, including IgG, IgM, IgE, IgA, IgD. Suitable antibodies are commercially available, for example from Abcam (Cambridge, UK).

30 The agent may be labelled to allow detection. Suitable detectable labels are known in the art. As an example, conjugation of an agent with horseradish peroxidase (HRP) allows detection of the agent by addition of an appropriate substrate that can be transformed by enzymatic action of HRP to produce a colour change. Other examples of detectable labels include alkaline phosphatase, peroxidase, colloidal gold, fluorescent 35 compounds, biotin, radioisotopes, luminescent compounds, magnetic particles, and

other enzymes. Detectable labels and labelling kits are commercially available from, for example, Expedeon Ltd, Cambridge, UK.

5 A primary/secondary antibody system may also be used to detect and/or determine the amount of biomarker in a sample. In such a system, a sample is contacted with a primary antibody that specifically recognizes and binds to one or more biomarkers. Subsequently, a secondary antibody with an appropriate label that recognizes the species or isotype of the primary antibody is contacted with the sample. Detection of the one or more biomarkers in the sample is achieved by measuring the signal
10 generated by a labelling molecule conjugated to the secondary antibody.

An antibody produced in a different species may be used for detection of a biomarker bound by a first antibody. For example, a first antibody generated in rabbit; and a secondary anti-species antibody able to recognise and bind the primary antibody
15 generated in a rabbit.

The agent may have the ability to bind to more than one biomarker, one or more of which are bound to a substrate. In such embodiments, the agents may be bound with spatial overlap. In some such embodiments the agents may be differentially labelled to
20 allow detection, for example the use of colour intensity-based labels allows detection and quantification of each spatially overlapping bound biomarker.

In some embodiments, the sample may be immobilised on a suitable substrate, such as a physical substrate such as a nitrocellulose or PVDF membrane, a rigid substrate made
25 of polystyrene, or other plastic polymer such as a microtiter plate. The substrate may then be contacted with an antibody that specifically recognises and binds to a first biomarker. Washing of the substrate helps to ensure specific binding of the antibody to the target biomarker. The antibody may be labelled, for example through conjugation with a detectable label such as an enzyme, fluorophore, or radioisotope. Detection of
30 the label and quantification of the amount of binding may be achieved by suitable means. Alternatively, a secondary antibody conjugated with a detectable label that binds to the biomarker-first antibody complex may be added, followed by washing to remove additional antibody and ensure that the presence of the detectable label on the substrate is indicative of the sample containing the biomarker being measured.

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Alternatively, a biomarker-specific antibody may be immobilised on the substrate, which is then contacted with a biomarker, itself being conjugated with a detectable label. Contact of the labelled biomarker with the antibody causes the antibody to bind the labelled biomarker. The sample is then contacted with the substrate in conditions
5 that allow binding of the biomarker to the antibody. Since the biomarker in the sample is not labelled, displacement of the labelled biomarker from the antibody results in a reduction in the amount of detectable label on the substrate after washing and indicates that the sample contains the biomarker.

10 Alternative agents include peptides, aptamers, enzymes, or small molecules that specifically bind to a target biomarker. For example, an aptamer that specifically binds H-FABP might be used. Aptamers are nucleic acid-based molecules that bind specific ligands. Methods for making aptamers with a particular binding specificity are known as detailed in U.S. Patent Nos. 5,475,096 ; 5,670,637 ; 5,696,249 ; 5,270,163 ;
15 5,707,796; 5,595,877 ; 5,660,985 ; 5,567,588 ; 5,683,867 ; 5,637,459 ; and 6,011,020.

Quantification of the amount of biomarker in a sample may be carried out using any suitable assay. For example, an antibody-based assay, or immunosorbent assay such as ELISA, radioimmunoassays or immunoprecipitation. In some embodiments, the
20 analysis is carried out using ELISA.

Alternative and/or complementary methods may be used to detect non-immunogenic molecules or compounds, such as metabolites, catabolites, peptides, hormones, signalling molecules, small molecules, drugs or any other compound or molecule that,
25 in its naturally occurring form, may not be recognised or bound by an antibody. Such methods include colorimetric methods, enzymatic methods, gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC) and high-performance liquid chromatography-mass spectrometry (HPLC-MS), nuclear magnetic resonance (NMR) spectroscopy, Raman
30 spectroscopy. For example, a suitable colorimetric method may be based upon a chemical reaction similar to that originally described by König [Journal fur Praktische Chemie, 1904, 70, 19-56 & Journal fur Praktische Chemie, 1904, 69, 105-137].

Moreover, techniques for measuring a biomarker *in vivo* include introducing a binding
35 agent that specifically recognises and binds the biomarker into a patient.

In some embodiments the amount of a biomarker in the sample taken from the individual identified as or suspected of having or having had a stroke is compared to a negative reference, and/or one or more positive references.

5 A negative reference is a laboratory solution such as a saline solution or buffer, where the target biomarker is absent, or the amount of the target biomarker is known to be undetectable. A positive reference is a laboratory solution such as a saline solution or buffer, comprising the target biomarker in a known amount. The biomarker used to create the positive and/or negative references may be the isolated target biomarker, or
10 a version thereof, such as a recombinant or synthetic version of the biomarker.

In some embodiments, the amount of biomarker in the sample taken from the individual identified as or suspected of having or having had a stroke may be compared to a negative reference and/or a range of positive references comprising different,
15 known amounts of the target biomarker.

In some embodiments a difference between the amount of biomarker in the sample taken from the individual identified or suspected as having or having had a stroke and the negative reference is indicative that the individual has suffered a stroke as a result
20 of LVO. In some embodiments, a statistically significant difference between the amount of biomarker in the sample taken from the individual identified or suspected as having or having had a stroke and the negative reference is indicative that the individual has suffered a stroke as a result of LVO. In some embodiments a correlation between the amount of biomarker in the sample taken from the individual identified or suspected as
25 having or having had a stroke and a positive reference determines or provides an indication of the amount of that biomarker in the sample from the individual.

Alternatively, or in addition, the amount of a biomarker in the sample taken from the individual identified or suspected as having or having had a stroke may be compared to
30 the amount of the same biomarker in a control.

A control is a sample or data generated from a sample taken from one or more healthy individuals. The term 'healthy individual' as used herein means an individual who has no signs or symptoms of stroke and who has no history of stroke. Alternatively or in
35 addition, the control may be a sample or data generated from a sample taken from one or more individuals who are having or who have had a TIA, stroke mimic,

haemorrhagic stroke or stroke resulting from small vessel occlusion. The term “individuals who are having or who have had a TIA, stroke mimic, haemorrhagic stroke or stroke resulting from small vessel occlusion” as used herein means individuals who are or have been determined, by clinical assessment, to be suffering a TIA, stroke
5 mimic, haemorrhagic stroke or stroke resulting from small vessel occlusion. Such patients may be included in the control group (i.e. contribute a sample for inclusion in the control) until neurological symptoms associated with the TIA, stroke mimic haemorrhagic stroke or stroke resulting from small vessel occlusion have ceased.

10 In some embodiments a difference between the amount of biomarker in the sample taken from the individual identified or suspected as having or having had a stroke and the control is indicative that the individual has suffered a stroke as a result of LVO. In some embodiments, a statistically significant difference between the amount of biomarker in the sample taken from the individual identified or suspected as having or
15 having had a stroke and the control is indicative that the individual has suffered a stroke as a result of LVO.

Alternatively or in addition, the amount of a biomarker in the sample from the individual may be compared to the amount of the same biomarker in a positive
20 standard. A positive standard is a sample or data generated from a sample taken from an individual who presents with one or more signs or symptoms associated with stroke who has, in addition, been diagnosed as having or having had a stroke as a result of LVO.

25 In some embodiments, a comparable amount of biomarker in the sample taken from the individual identified as or suspected of having or having had a stroke and the positive standard is indicative that the individual has suffered a stroke as a result of LVO. In some embodiments, a statistically significant difference between the amount of biomarker in the sample taken from the individual identified as or suspected of having
30 or having had a stroke and the positive standard is indicative that the individual has suffered a stroke as a result of LVO.

In some embodiments, a ratio between two or more biomarkers may be calculated, wherein the ratio is the amount of one biomarker (the ‘target biomarker’) relative to the
35 amount of the other biomarker (the ‘reference biomarker’). In some embodiments, the reference biomarker is in a control, a positive reference or a positive standard; for

example, the amount of biomarker in a sample taken from an individual suspected of having a LVO stroke may be compared to a sample or data generated from a sample taken from one or more healthy individuals. In some embodiments the target and reference biomarkers are from the same sample, for example the amount of succinate
5 may be compared to the amount of GFAP in a sample taken from an individual suspected of having a LVO stroke. Alternatively or in addition, the level of a biomarker(s) may be analysed in samples taken from the same individual at different times, for example, the amount of GFAP may be compared in two samples taken from an individual before and after the individual has suffered a stroke. In some
10 embodiments a positive ratio is indicative that the individual has suffered a stroke as a result of LVO, whilst a negative ratio is indicative that the individual has not suffered a stroke as a result of LVO, wherein the phrase “a positive ratio” denotes an increased amount of a test biomarker relative to a reference biomarker; and “a negative ratio” refers to a decreased amount of a test biomarker relative to the amount of a reference
15 biomarker. A neutral ratio describes no difference or effectively no difference in the amounts of the test and reference biomarker.

In some embodiments, one or more of the measured biomarker amount(s) in the sample are transformed into an LVO stroke score. In some embodiments, the LVO
20 stroke score involves correlating the amount of a biomarker in the sample from the individual with one or more of a negative reference, a positive reference, a control or a positive standard. For example, an LVO stroke score from 0 to 100 may be established, where 0 corresponds to the value of the biomarker in a control, and 100 corresponds to the value of the biomarker in a positive standard. A weight factor may be assigned to
25 one or more of the biomarkers. For example, the biomarkers may be weighted such that biomarkers with a higher diagnostic relevance contribute more to the LVO stroke score. For example, a biomarker may be of higher diagnostic relevance if the amount of that biomarker differs significantly between an individual diagnosed as having or having had an LVO and a control. In contrast, a biomarker may be of less diagnostic relevance
30 if the difference in the amount of that biomarker in an individual diagnosed as having or having had an LVO and a control is small. Such weight factors modulate the contribution of the biomarker to the diagnosis of LVO stroke. The weight factor may be introduced in a computational step, for example using an algorithm, or when binding of an agent to the biomarker is detected and measured in a detection tool such as an
35 optical reader.

In some embodiments, the LVO stroke score created using the measured biomarker amounts in a sample taken from an individual identified as or suspected of having or having had a stroke may be compared to an LVO stroke score created using biomarker amounts from a control, or from a positive standard.

5

In some embodiments, an LVO score created using measured biomarker amounts in a sample taken from the individual identified as or suspected of having or having had a stroke which is comparable to an LVO score created using biomarker amounts from a positive standard is indicative that the individual has suffered a stroke as a result of LVO. In some embodiments, an LVO score created using measured biomarker amounts in a sample taken from the individual identified as or suspected of having or having had a stroke which is statistically comparable to an LVO score created using biomarker amounts from a positive standard is indicative that the individual has suffered a stroke as a result of LVO.

15

In some embodiments, a difference between an LVO score created using measured biomarker amounts in a sample taken from the individual identified as or suspected of having or having had a stroke and a control is indicative that the individual has suffered an LVO stroke. In some embodiments, a statistically significant difference between an LVO score created using measured biomarker amounts in a sample taken from the individual identified as or suspected of having or having had a stroke and a control is indicative that the individual has suffered an LVO stroke.

25

In some embodiments, a defined value or range ("cut-off value") is provided for one or more of the biomarkers, and/or for the LVO stroke score. In some such embodiments, an LVO stroke score or an amount of a biomarker above the defined value is indicative that the individual has suffered a stroke resulting from LVO. In other such embodiments, an LVO stroke score, or an amount of a biomarker below the defined value is indicative that the individual has not suffered a stroke resulting from LVO.

30

In some embodiments, the method may be carried out within about 72, within about 48 or within about 24 hours of the onset of stroke symptoms in the individual. In some embodiments, the method is carried out within about 24 or within 24 hours of the onset of stroke symptoms in the individual.

35

In some embodiments, the method further comprises consideration of data and/or information from one or more clinical assessments the individual has undergone. The clinical assessments may include one or more of CT or CTA, MRA, MRI, diffusion weighted imaging, cerebral angiography, electrocardiogram, atrial fibrillation
5 assessment, FAST (face, arm, speech test), ABCD, ABCD2 and California prediction rules Rosier (Recognition of Stroke in the Emergency Room) stroke scale, stroke scale, and TOAST (trial of ORG. 10172 in Acute Stroke Treatment) classification, OCSF (the Oxford Community Stroke Project classification), National Institutes of Health Stroke Scale (NIHSS), Emergency Medical Stroke Assessment (EMSA), Prehospital Acute
10 Stroke Severity (PASS) scale, Vision, Aphasia, Neglect (VAN) assessment, Rapid Arterial Occlusion Evaluation (RACE), Field Assessment Stroke Triage for Emergency Destination (FAST-ED) scale, Cincinnati pre-hospital Stroke Severity Scale (CPSSS), doppler ultrasound, carotid duplex ultrasound, angiography or arteriography. In some embodiments, the method further comprises consideration of data and/or information
15 from angiographical assessment by CTA and/or MRA.

CTA is a computer-reconstructed tomography technique used to visualise arterial and venous vessel at the level of the head, neck, and/or brain. By applying a contrast agent, CTA allows the visualisation of blockages, aneurysms, dissections and stenosis of blood
20 vessels. Contrast agents are any radiodense compounds that can block the X-ray portion of the electromagnetic spectrum, therefore resulting into opacity areas of the computed images.

MRA refers to any magnetic resonance imaging-based diagnostic technique used to
25 generate images of blood vessels to visualise blockages, aneurysms, dissections and stenosis of blood vessels. Contrast agents, such as the ones based on gadolinium are applied to generate the images and a variety of different techniques are available (Johnson et al "Magnetic resonance angiography: A review." Academic radiology 5, no. 4 (1998): 289-305.)

30

Identification of an individual who is suffering or has suffered a stroke as a result of LVO allows selection of appropriate further clinical investigations and or a treatment pathway for that individual.

35 Accordingly in a second aspect there is provided a method of identifying, from a group of individuals identified as or suspected of having or having had a stroke, individuals

who have suffered a stroke as a result of LVO, comprising determining the amount of at least two biomarkers in a sample taken from each individual wherein the biomarkers are selected from: succinate, succinic-glutathione, N-acetyl-aspartate, propionyl-carnitine, glutamate, heart fatty acid binding protein (H-FABP), brain fatty acid
5 binding protein (B-FABP), Abeta 1-40, OPG, soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK), pro-vWF, retinol binding protein 4 (RBP4), ADAMTS13, NMDA receptor 2 peptide (NR2 peptide), 20-HETE, bilirubin, brain natriuretic peptide (BNP), glial fibrillary acidic protein (GFAP), D-dimer, C-reactive Protein (CRP), matrix metallo- proteinase 9 (MMP9), interleukin 6 (IL-6), osteopontin
10 (OPN), Troponin I, s100b, von Willebrand Factor (vWF) or P-selectin.

In some embodiments the method according to the second aspect comprises determining the amount of at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least 10, at least eleven, or 12 or more biomarkers.

15 The method according to the second aspect comprises determining the presence and/or amount of one or more biomarkers in accordance with any embodiment of the first aspect.

20 In some embodiments according to the second aspect the method comprises comparing the amount of each biomarker in the sample taken from each individual identified as or suspected of having or having had a stroke to the amount of the same biomarker in one or more of a control, a positive standard, one or more positive references or a negative reference, as disclosed in accordance with the first aspect, above.

25 In some embodiments according to the second aspect, individuals with a difference in the amount of one or more of the measured biomarker relative to the amount of the same biomarker in a control are identified as having or having had a stroke as a result of LVO. In some embodiments according to the second aspect, individuals with a
30 comparable amount of one or more of the measured biomarker relative to a positive standard are identified as having or having had a stroke as a result of LVO.

In some embodiments the method comprises transforming the amount of one or more biomarkers measured in the sample taken from an individual identified as or suspected
35 of having or having had a stroke into an LVO stroke score, as disclosed in accordance with the first aspect, above.

In some embodiments according to the second aspect, individuals with an LVO score comparable to an LVO score created using biomarker amounts from a positive standard are identified as having or having had a stroke as a result of LVO. In some
5 embodiments, individuals with an LVO score statistically comparable to an LVO score created using biomarker amounts from a positive standard are identified as having or having had a stroke as a result of LVO. In some embodiments, individuals with an LVO score that differs from an LVO score created using biomarker amounts from a control are identified as having or having had a stroke as a result of LVO. In some
10 embodiments, individuals with an LVO score that is statistically different from an LVO score created using biomarker amounts from a control are identified as having or having had a stroke as a result of LVO.

In some embodiments the method according to the second aspect further comprises
15 consideration of data and/or information from one or more clinical assessments which each individual has undergone, as disclosed in accordance with the first aspect, above. In some such embodiments the clinical assessment is one or more of a CT or CTA, MRA or MRI scan, or the individual's NIHSS, FAST, ABCD, ABCD2 Rosier, TOAST, EMSA, PASS, VAN, RACE, FAST-ED or CPSS score.

20 Appropriate further clinical investigations for an individual who is identified as suffering from or has suffered a stroke as a result of LVO include computerised tomography angiography and/or magnetic resonance angiography. Appropriate treatment pathways for an individual who is identified as suffering from or has suffered
25 a stroke as a result of LVO may include treatment with antithrombotic agents and/or mechanical thrombectomy.

In accordance with a third aspect, there is provided the use of two or more biomarkers to diagnose stroke resulting from large vessel occlusion, wherein the biomarkers are
30 selected from succinate, succinic-glutathione, N-acetyl-aspartate, propionyl-carnitine, glutamate, heart fatty acid binding protein (H-FABP), brain fatty acid binding protein (B-FABP), Abeta 1-40, OPG, soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK), pro-vWF, retinol binding protein 4 (RBP4), ADAMTS13, NMDA receptor 2 peptide (NR2 peptide), 20-HETE, bilirubin, brain natriuretic peptide (BNP),
35 glial fibrillary acidic protein (GFAP), D-dimer, C-reactive Protein (CRP), matrix

metallo- proteinase 9 (MMP9), interleukin 6 (IL-6), osteopontin (OPN), Troponin I, s100b, von Willebrand Factor (vWF) or P-selectin.

5 In some embodiments, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least 10, at least eleven, or 12 or more biomarkers are used to identify stroke resulting from large vessel occlusion.

10 In some embodiments, the biomarkers are in a sample taken from an individual identified as or suspected of having or having had a stroke. The biomarkers may be detected using any method disclosed herein. In addition, the amount of biomarker may be determined using any method disclosed herein.

15 There is also provided, in a fourth aspect, a kit for carrying out a method according to the first of second aspects of the invention. The kit may comprise one or more of: a container for holding a sample from the individual identified or suspected as having or having had a stroke; a means for determining the amount of the target biomarker(s); and instructions, such as printed instructions on how to carry out the reaction between the sample and the means provided so as to determine the amount of the target biomarker(s).

20 In some embodiments, the means for determining the amount of the target biomarker(s) may comprise one or more agents as disclosed herein. The agent(s) may be antibodies. The agents may be immobilised on a substrate and/or may be labelled with at least one detectable label. In some embodiments each agent is labelled with a different detectable label.

25

Without wishing to be bound by any theory, it is suggested that the accuracy of detection of stroke resulting from LVO in methods according to the invention arises as a result of the detection of certain amounts of specific biomarkers or a specific combination of biomarkers in a sample from an individual suspected of having or having had a stroke, levels of which are altered as a result of biological changes that occur as a result of LVO stroke. It is suggested that such markers are associated with brain ischemia and/or death of brain cells, both of which are increased in LVO in comparison to other types of stroke. Ischemia can result in damage to tissue and cells resulting in release and/or upregulation of biomarkers either from the damaged cells or tissues themselves, or from tissues or cells in communication with injured cells.

30

35

Accordingly, some embodiments of the invention provide methods, kits and uses for identifying or helping to identify those individuals presenting with stroke who are or have suffered a stroke resulting from LVO. Such embodiments offer the opportunity to
5 allow determination of such individuals with improved accuracy and/or speed, thereby improving the treatment pathway for the individual.

The following examples are provided to illustrate the present invention and should not be construed as limiting thereof.

10

Example 1

Example 1A: Sample Preparation

A sample of blood may be withdrawn via venepuncture from a human subject and collected into tubes containing sodium EDTA and immediately placed on ice.

15 Alternatively, a sample of CSF may be obtained by lumbar puncture.

The need for and type of processing of the sample prior to biomarker detection depends upon the biomarker to be detected and/or the assay to be used to detect the biomarker. Where whole blood is required for analysis, the sample can be used as obtained.

20 Alternatively, the sample can be diluted with saline solution to obtain an appropriate concentration of biomarkers that are compatible with the required type of biomarker detection assay.

If blood plasma is required for analysis, the whole blood sample can be centrifuged to
25 obtain the plasma, for example at 2000 g for 15 minutes at 4°C. The plasma can then be drawn off and used directly, or diluted with saline solution as required for the biomarker detection assay.

Example 1b: Optimisation of the Assay

30 Rabbit monoclonal antibodies are purchased from Abcam, Cambridge UK. A known concentration of isolated or recombinant antibody is applied in a preliminary assay to determine the specificity of antibodies to recognise and bind the target biomarker. To determine optimal concentration of the antibodies an indirect ELISA is performed wherein the isolated or recombinant biomarker protein is attached to an ELISA
35 microtiter plate. Rabbit monoclonal anti-human biomarker antibody is coated to the microtiter plate to determine the concentration of antibody necessary to obtain a

maximum signal, allowing determination of the lower limit of detection of indirect ELISA for each antibody. After incubation of a diluted sample with a rabbit monoclonal antihuman biomarker antibody for 2 hours, and appropriate wash is performed, a monoclonal anti-human biomarker antibody labelled with biotin is then added and
5 incubated with captured biomarker. Multiple steps of washing are then performed, after which horseradish peroxidase conjugated with streptavidin is added. Incubation of the reagents for one hour is then followed by other washing steps, after which the generated conjugate is contacted with hydrogen peroxide tetramethyl benzadine, producing a yellow product. The reaction is stopped by adding acidic solution and
10 reaction signal mis-measured by absorbance at 450 nanometers, with the absorbance being proportional to the concentration of the biomarker. By plotting absorbance values as a function of measured biomarker quantities in samples at known biomarker concentrations a standard curve is obtained. The standard curve is then used to determine the quantity of target biomarker in unknown samples.

15

Example 1C: Materials for Biomarker Analyses

Illustrative reagents used in performing the subject invention include Sodium bicarbonate (Sigma Cat #: C-3041), blocking buffer (Startingblock T20-TBS) (Pierce Cat#: 37543), Tris buffered saline with Tween 20 (TBST; Sigma Cat #: T-9039).
20 Phosphate buffered saline (PBS; Sigma Cat #: P-3813); Tween 20 (Sigma Cat #: P5927); Ultra TMB ELISA (Pierce Cat #: 34028); and Nunc maxisorp ELISA plates (Fisher). Monoclonal and polyclonal antibodies against heart fatty acid binding protein (H-FABP), brain fatty acid binding protein (B-FABP), Abeta 1-40, OPG, soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK), pro-vWF, retinol binding
25 protein 4 (RBP4), ADAMTS13, NMDA receptor 2 peptide (NR2 peptide), 20-HETE, brain natriuretic peptide (BNP), glial fibrillary acidic protein (GFAP), D-dimer, C-reactive Protein (CRP), matrix metallo- proteinase 9 (MMP9), interleukin 6 (IL-6), osteopontin (OPN), Troponin I, s100b, von Willebrand Factor (vWF), P-selectin are purchased from Abcam, Cambridge, UK. Labels for antibodies of numerous subtypes
30 are available from Expedeon Ltd, Cambridge, UK. Protein concentrations in samples are determined using bicinchoninic acid microprotein assays (Pierce Inc., Rockford, IL, USA) with albumin standards. All other necessary reagents and materials will be known to those of skill in the art and are readily ascertainable. Colorimetric assay kits for bilirubin, succinate, N-acetylaspartate, Propionyl-L-carnitine, glutamate are available
35 from Abcam, Cambridge, UK and Biovision Inc, Milpitas, CA.

In order to address various issues and advance the art, the entirety of this disclosure shows by way of illustration various embodiments in which the claimed invention(s) may be practiced and provide for a superior diagnostic test for stroke resulting from large vessel occlusion. The advantages and features of the disclosure are of a
5 representative sample of embodiments only, and are not exhaustive and/or exclusive. They are presented only to assist in understanding and teach the claimed features. It is to be understood that advantages, embodiments, examples, functions, features, structures, and/or other aspects of the disclosure are not to be considered limitations on the disclosure as defined by the claims or limitations on equivalents to the claims,
10 and that other embodiments may be utilised and modifications may be made without departing from the scope and/or spirit of the disclosure. Various embodiments may suitably comprise, consist of, or consist essentially of, various combinations of the disclosed elements, components, features, parts, steps, means, etc. In addition, the disclosure includes other inventions not presently claimed, but which may be claimed
15 in future.

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Claims

1. A method of diagnosing stroke as a result of a large vessel occlusion (LVO), comprising determining the amount of at least two biomarkers in a sample taken from
5 an individual identified as or suspected of having or having had a stroke, wherein the biomarker(s) are selected from: succinate, succinic-glutathione, N-acetyl-aspartate, propionyl-carnitine, glutamate, heart fatty acid binding protein (H-FABP), brain fatty acid binding protein (B-FABP), Abeta 1-40, OPG, soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK), pro-vWF, retinol binding protein 4 (RBP4),
10 ADAMTS13, NMDA receptor 2 peptide (NR2 peptide), 20-HETE, bilirubin, brain natriuretic peptide (BNP), glial fibrillary acidic protein (GFAP), D-dimer, C-reactive Protein (CRP), matrix metallo- proteinase 9 (MMP9), interleukin 6 (IL-6), osteopontin (OPN), Troponin I, s100b, von Willebrand Factor (vWF) or P-selectin.
- 15 2. A method of identifying, from a group of individuals identified as or suspected of having or having had a stroke, individuals who have suffered a stroke as a result of LVO, comprising determining the amount of at least two biomarkers in a sample taken from each individual, wherein the biomarker(s) are selected from: succinate, succinic-glutathione, N-acetyl-aspartate, propionyl-carnitine, glutamate, heart fatty acid binding
20 protein (H-FABP), brain fatty acid binding protein (B-FABP), Abeta 1-40, OPG, soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK), pro-vWF, retinol binding protein 4 (RBP4), ADAMTS13, NMDA receptor 2 peptide (NR2 peptide), 20-HETE, bilirubin, brain natriuretic peptide (BNP), glial fibrillary acidic protein (GFAP), D-dimer, C-reactive Protein (CRP), matrix metallo- proteinase 9 (MMP9), interleukin 6
25 (IL-6), osteopontin (OPN), Troponin I, s100b, von Willebrand Factor (vWF) or P-selectin.
3. The method of claim 1 or claim 2, comprising determining the amount of at least three, at least four, at least five, at least six, at least seven, at least eight, at least
30 nine, at least 10, at least eleven, or 12 or more biomarkers.
4. The method of any one of claims 1-3, comprising comparing the amount of each biomarker in the sample taken from the individual identified as or suspected of having or having had a stroke to the amount of the same biomarker in one or more of a control,
35 a positive standard, one or more positive references or a negative reference.

5. The method of claim 4, wherein the individual is diagnosed as having or having had a stroke as a result of LVO when the amount of one or more of the measured biomarkers differs from the amount of the same biomarker in a control; or is comparable to the amount of the same biomarker in a positive standard.
- 5 6. The method of any one of claims 1-4, comprising transforming the amount of one or more biomarkers measured in the sample taken from an individual identified as or suspected of having or having had a stroke into an LVO stroke score.
- 10 7. The method of claim 6, comprising comparing the LVO stroke score created using the measured biomarker amounts in a sample taken from an individual identified as or suspected of having or having had a stroke to an LVO stroke score created using biomarker amounts from a control, or from a positive standard.
- 15 8. The method of claim 7, wherein the individual is diagnosed as having or having had a stroke as a result of LVO when the LVO stroke score created using the measured biomarker amounts in a sample taken from an individual identified as or suspected of having or having had a stroke is comparable to an LVO stroke score created using biomarker amounts from a positive standard; or when the LVO stroke score created
- 20 using the measured biomarker amounts in a sample taken from an individual identified as or suspected of having or having had a stroke differs from the LVO stroke score created using biomarker amounts from a control.
9. The method of any one of claims 1-8, wherein the sample from the individual is
- 25 one or more of blood, plasma, cerebrospinal fluid or saliva.
10. The method of any one of claims 1-9, further comprising considering data and/or information from one or more clinical assessments which the individual has undergone, preferably wherein the clinical assessments are one or more of a CT or CTA
- 30 scan, MRA or MRI scan, or the individual's NIHSS, FAST, ABCD, ABCD2 Rosier, TOAST, EMSA, PASS, VAN, RACE, FAST-ED or CPSS score.
11. A method as claimed in any one of claims 1-10, wherein the amount of biomarker in the sample taken from the individual identified or suspected as having or
- 35 having had a stroke is determined using an agent that binds to the biomarker; preferably wherein the amount of biomarker is determined using ELISA.

12. The method of any one of claims 1-11, wherein the method is carried out within about 24 hours of onset of stroke symptoms in the individual.

5 13. Use of two or more biomarkers to diagnose stroke resulting from large vessel occlusion (LVO), wherein the biomarkers are selected from: succinate, succinic-glutathione, N-acetyl-aspartate, propionyl-carnitine, glutamate, heart fatty acid binding protein (H-FABP), brain fatty acid binding protein (B-FABP), Abeta 1-40, OPG, soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK), pro-vWF, retinol
10 binding protein 4 (RBP4), ADAMTS13, NMDA receptor 2 peptide (NR2 peptide), 20-HETE, bilirubin, brain natriuretic peptide (BNP), glial fibrillary acidic protein (GFAP), D-dimer, C-reactive Protein (CRP), matrix metallo- proteinase 9 (MMP9), interleukin 6 (IL-6), osteopontin (OPN), Troponin I, s100b, von Willebrand Factor (vWF) or P-selectin.

15 14. The use as claimed in claim 13, wherein at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, at least eleven or twelve or more of the biomarkers are used to identify stroke resulting from large vessel occlusion in an individual identified or suspected as having or having had a stroke.

20 15. A kit for carrying out a method according any of claims 1-12, comprising one or more of: a container for holding a sample from the individual identified or suspected as having or having had a stroke; a means for determining the amount of the target biomarker; and instructions on how to carry out the reaction between the sample and
25 the means provided so as to determine the amount of the target biomarker(s).



Application No: GB2005632.1

Examiner: Katie Harbach

Claims searched: 1-15 in part

Date of search: 29 October 2020

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
Y	1-15	WO99/42607 A1 (UNIV NOTRE DAME DU LAC) See particularly page 5 line 23 to page 6 line 3.
Y	1-15	Biochimica et Biophysica Acta - Molecular Basis of Disease, vol. 1863, no.9, 2017, Wu et al., "Succinate-induced neuronal mitochondrial fission and hexokinase II malfunction in ischemic stroke: Therapeutical effects of kaempferol", pages 2307-2318. See especially discussion paragraphs 1 and 2. Available from: https://www.sciencedirect.com/science/article/pii/S0925443917302065?via%3Dihub#s0005 [Accessed 28.10.2020].
Y	1-15	Nature, vol 515, 2014, Couchani et al, "Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS.", pages 431-435. See whole document . Available from: https://www.nature.com/articles/nature13909 [Accessed 28.10.2020].
Y	1-15	WO2012/009567 A2 (UNIV CALIFORNIA ET AL) See particularly paragraph [0007].
Y	1-15	WO2016/087611 A1 (UNIV VALL HEBRON INST) See page 2 line 24 to page 4 line 9.

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International Classification:

Subclass	Subgroup	Valid From
G01N	0033/68	01/01/2006
G01N	0033/50	01/01/2006
G01N	0033/53	01/01/2006