

BLOOD BIOMARKERS OF STROKE

FIELD OF INVENTION

The present invention relates to blood biomarkers of stroke and uses thereof in particular
5 for monitoring the efficiency of therapies in ischemic stroke, for diagnosing purposes, short- and long-term prognosis as well as for risk assessment.

BACKGROUND OF INVENTION

Stroke is the second leading cause of death worldwide and the third leading cause of
10 disability (McKay & Mensah, **2005**. *The atlas of heart disease and stroke* (1st ed.). Geneva: World Health Organization). Cerebral focal ischemia, *i.e.*, ischemic stroke, leads to severe and rapid tissue injury at the core of the infarction. After initial injury, brain cell death progresses slowly, extending to a heterogeneous area surrounding the core called the penumbra (Astrup *et al.*, **1977**. *Stroke*. **8(1)**:51-7). Salvaging the ischemic penumbra
15 improves outcomes in terms of disability (Emberson *et al.*, **2014**. *Lancet*. **384(9958)**:1929-35).

To date, the only emergency therapeutic solution is recanalization through thrombolysis and/or thrombectomy. However, due to the short intervention window (< 6 hours for recanalization and 4h30 for thrombolysis) and haemorrhagic transformation risk, only a
20 small percentage of acute ischemic stroke patients are eligible for this treatment. For example, it is estimated that among all patients arriving within 6 hours at a comprehensive stroke centre, only 10.5% are endovascular-eligible for thrombectomy according to AHA/ASA criteria (Vanacker *et al.*, **2016**. *Stroke*. **47(7)**:1844-9).

Therefore, developing a new therapeutic strategy that widens the therapeutic window is
25 a major public health issue. While numerous clinical trials failed before, since the advent of recanalization, neuroprotection associated to recanalization remains a potentially promising strategy to widen the therapeutic window. Increasing evidences suggest that peripheral proteins, nucleic acids, or lipids can be used to confirm diagnosis of ischemic

stroke and to monitor disease progression. To date, however, none has been implemented in clinical practice (Kim *et al.*, **2013**. *J Stroke*. **15**(1):27-37).

Biomarker tests refer to imaging, chemical, or other biological tests that can be used to qualitatively assess or quantitatively measure the presence or absence of one or several markers, indicative of the presence, progress or severity of a disease, or of the effects of a treatment. Central nervous system (CNS) imaging has made tremendous progresses, but identification of peripheral biological markers, on the other hand, is less advanced. Identifying blood biomarkers to be applied as robust biomarkers for CNS diseases and treatment is one of the most important challenges in modern neurology, as blood lacks direct contact with the brain.

The use of RNA in the blood as a diagnostic marker is an emerging field that is supported by its clinical application in the diagnosis of breast cancer, coronary artery disease and infectious disease (Rothstein & Jickling, **2013**. *Biomark Med*. **7**(1):37-47). Furthermore, RNA in the blood could be used as a companion diagnostic to assess the effect of a neuroprotective agent in early clinical trials. Studies of RNA as a diagnostic biomarker in acute ischemic stroke are rare, including only a small number of patients, among which only few patients at the acute phase of infarction (Tang *et al.*, **2006**. *J Cereb Blood Flow Metab*. **26**(8):1089-102; Stamova *et al.*, **2010**. *Stroke*. **41**(10):2171-7) for coding RNA. Recently, there has been burgeoning specific interest in non-coding RNA as potential biomarkers in stroke as a risk factor, but not at the acute phase of stroke infarction (Mick *et al.*, **2017**. *Stroke*. **48**(4):828-834).

Transcriptome analysis has been used in many experimental studies with cerebral ischemia to measure gene expression changes. Most experimental transcriptomic studies were performed in rat and mouse on focal or global ischemia models (for a review, see Cox-Limpens *et al.*, **2014**. *Brain Res*. **1564**:85-100). Besides ischemia, preconditioning has also been used as a tool to study endogenous brain protection in rodents. Results of microarray studies examining the transcriptome in these rodents have shown that immediate early genes, stress response genes, apoptosis genes, signal transduction genes, neurotransmission genes, ion channels genes, inflammation genes, cytoskeleton genes, ribosomal genes, and neurotrophic factors genes undergo expression changes during

cerebral ischemia (Schmidt-Kastner *et al.*, **2002**. *Brain Res Mol Brain Res*. **108(1-2)**:81-93; Büttner *et al.*, **2009**. *Brain Res*. **1252**:1-14; Wang *et al.*, **2011**. *Brain Res*. **1372**:13-21; Wang *et al.*, **2012**. *Neuroscience*. **220**:100-8; Lu *et al.*, **2004**. *J Neurosci Res*. **77(6)**:843-57). However, to our knowledge, only few experiments were performed in
5 primates (Cook *et al.*, **2012**. *Nature*. **483(7388)**:213-7; Cook & Tymianski, **2012**. *Neurotherapeutics*. **9(2)**:371-9). Primates represent unique models to study brain ischemia since they have a highly similar genomes to human, as well as anatomical homology. For example, both primates and humans have non-lissencephalic brains (*i.e.*, which exhibit convolutions in the cortex; gyrencephalic) as opposed to rodents, which do
10 not. Furthermore, the transcriptomes of the cerebral cortex, in both human and chimpanzee, are very similar to each other and differ more between individuals than among regions within an individual (Khaitovich *et al.*, **2004**. *Genome Res*. **14(8)**:1462-73).

Four main reasons for the lack of success in translating neuroprotective therapies from
15 animal studies to application in human have been identified (Moretti *et al.*, **2015**. *Pharmacol Ther*. **146**:23-34; Timsit & Menn, **2012**. *Clin Pharmacol Ther*. **91(2)**:327-32):

- i) the poor quality of preclinical studies:
for preclinical studies, the challenge in this field is being progressively overcome through the development of quality score criteria in preclinical studies, *e.g.*, the
20 STAIR criteria (Stroke Therapy Academic Industry Roundtable (STAIR), **1999**. *Stroke*. **30(12)**:2752-8; Fisher *et al.*, **2009**. *Stroke*. **40(6)**:2244-50); and of randomized blind studies in human (Llovera *et al.*, **2015**. *Sci Transl Med*. **7(299)**:299ra121).
- ii) the absence of biologic companion pharmacodynamics biomarkers in early clinical
25 development:
companion biomarkers to develop drug is now more and more used. To gain confidence as to whether efficacy can be achieved in diseased patients with safe doses, prior to the big investments required in later stages, relatively short duration phase Ib clinical studies are conducted in small numbers of patients having the

relevant disease. This is the most important application of some of the pharmacodynamic biomarkers (Zhao *et al.*, **2015**. *Clin Chem.* **61**(11):1343-53).

iii) the absence of predictive imaging biomarkers in phase II human studies to estimate the penumbra:

5 selection of patients based on cerebral imaging to identify patients suitable to thrombectomy is now instrumental, as shown in the DAWN study to save the penumbra in selected patients (Jovin *et al.*, **2017**. *Int J Stroke.* **12**(6):641-652; Chaisinanunkul *et al.*, **2015**. *Stroke.* **46**(8):2238-43; Nogueira *et al.*, **2018**. *N Engl J Med.* **378**(1):11-21). Only a few studies in neuroprotection have carried out
10 selection on the basis of penumbral characteristics (for a review, see Donnan *et al.*, **2009**. *Lancet Neurol.* **8**(3):261-9). Today, this novel concept may offer a useful avenue for therapeutic intervention (Hillis & Baron, 2015. *Front Neurol.* **6**:85) while waiting for recanalization or reperfusion.

iv) the absence of recanalization *in fine*:

15 thrombectomy is becoming the standard treatment at the acute phase of ischemic stroke, although its use will need to be spread. It is estimated that among all patients arriving within 6 hours at a comprehensive stroke centre, only 10.5% are “endovascular-eligible” for thrombectomy according to AHA/ASA criteria (Vanacker *et al.*, **2016**. *Stroke.* **47**(7):1844-9).

20 A better understanding of gene expression patterns in tissue undergoing cerebral ischemia can improve diagnostic techniques. Here, the Inventors have explored the expression of genes after the onset of cerebral ischemia in the brain and blood. The study of macaque microarray expression data from brain and blood revealed that ischemic and non-ischemic samples can be distinguished based on their expression profiles, and the majority of
25 highly differentially expressed genes are up-regulated in the ischemic scenario 6 hours after ischemia. A comparison of genes differentially expressed in the brain and the blood revealed a significant overlap of gene expression patterns. Surprisingly, the data showed that there is a common coding signature between the ischemic brain and the blood, which supports the development of blood transcriptomics as a tool for biopsy transcriptome

expression profiling to characterize patients with ischemic stroke, to develop a companion biomarker for the assessment of neuroprotection drugs in patients.

SUMMARY

- 5 The present invention relates to a method of diagnosing a stroke in a subject, comprising:
- i) determining a signature in a sample obtained from the subject by measuring the expression levels of at least two biomarkers selected from the group consisting of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*, preferably with the proviso that the at least two biomarkers do not consist in *DUSP1*
10 and *ADM*;
 - ii) comparing the signature determined in step i) with a reference signature; and
 - iii) diagnosing the subject as being affected with a stroke when the expression levels of the at least two biomarkers in the signature are higher than the expression levels of the same at least two biomarkers in the reference signature.

- 15 In one embodiment, step i) of the method of diagnosing a stroke in a subject comprises measuring the expression levels of at least three biomarkers selected from the group consisting of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*.

- In one embodiment, step i) of the method of diagnosing a stroke in a subject comprises
20 measuring the expression levels of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*.

In one embodiment, the reference signature is obtained by measuring the expression levels of the biomarkers in a reference population of substantially healthy subjects.

- In one embodiment, the method of diagnosing a stroke in a subject according to the present
25 invention aims at distinguishing a stroke from a stroke mimic.

The present invention further relates to a method of determining whether a subject suffering from a stroke will achieve a response with a therapy, comprising:

- i) determining a signature in a sample obtained from the subject by measuring the expression levels of at least two biomarkers selected from the group consisting of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*; preferably with the proviso that the at least two biomarkers do not consist in *DUSP1* and *ADM*
- ii) comparing the signature determined in step i) with a reference signature; and
- iii) concluding that the subject achieves a response when the expression levels of the at least two biomarkers in the signature are lower than the expression levels of the same at least two biomarkers in the reference signature.

10 In one embodiment, step i) of the method of determining whether a subject suffering from a stroke will achieve a response with a therapy comprises measuring the expression levels of nine biomarkers selected from *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*.

15 In one embodiment, the reference signature is obtained by measuring the expression levels of the biomarkers in a sample obtained from the same subject before the start of said therapy.

The present invention relates to a method of determining whether a subject is at risk of having a stroke, comprising:

- i) determining a signature in a sample obtained from the subject by measuring the expression levels of at least two biomarkers selected from the group consisting of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*; preferably with the proviso that the at least two biomarkers do not consist in *DUSP1* and *ADM*
- ii) comparing the signature determined in step i) with a reference signature; and
- 25 iii) concluding that the subject is at risk of having stroke when the expression levels of the at least three biomarkers in the signature are higher than the expression levels of the same at least three biomarkers in the reference signature.

In one embodiment, step i) of the method of determining whether a subject is at risk of having a stroke comprises measuring the expression levels of nine biomarkers selected from *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*.

5 In one embodiment, the reference signature is obtained by measuring the expression levels of the biomarkers in a reference population of substantially healthy subjects.

In one embodiment, the subject has experienced a stroke and the method of determining whether a subject is at risk of having a stroke is for determining if the subject is at risk of having a recurrent stroke.

10 In one embodiment pertaining to any of the methods of the invention, stroke is ischemic stroke, transient ischemic attack or a haemorrhagic stroke.

In one embodiment pertaining to any of the methods of the invention, the sample is a blood sample, plasma sample or serum sample.

15 In one embodiment pertaining to any of the methods of the invention, the sample is not a brain sample.

DEFINITIONS

In the present invention, the following terms have the following meanings:

20 “**Stroke**”, as used herein, refers to any condition arising from a disruption, decrease, or stoppage of blood or oxygen flow to any part of the brain. In particular, the term “**stroke**” encompasses, without limitation, ischemic stroke, transient ischemic attack (TIA) and haemorrhagic stroke.

25 “**Ischemic stroke**” (or “**IS**”) is an episode of neurological dysfunction caused by focal brain, spinal cord, or retina ischemia with evidence of acute infarction (Easton *et al.*, 2009. **Stroke**. 40(6):2276-2293). There are at least four different causes of blood flow interruption:

- (1) a blood clot in a blood vessel;
- (2) a blood clot in the dural venous sinuses, which drain blood from the brain;

(3) an embolus clogging a blood vessel; and/or

(4) a sudden drop in blood pressure.

Stroke symptoms can, and frequently do, persist beyond 24 hours if the patient survives the initial damage.

- 5 “**Transient ischemic attack**” or “**TIA**”, also called a mini-stroke, is a transient episode of neurological dysfunction caused by focal brain, spinal cord, or retinal ischemia without evidence of acute infarction. TIA symptoms can initially be the same as a stroke, except that the symptoms only last a short time, typically less than one hour, or at most 24 hours. Even though a TIA is temporary and usually does not cause brain tissue damages, a
- 10 patient experiencing TIA is advised to seek professional help immediately because of the similarity in symptoms and because TIA is a risk factor for subsequent ischemic strokes.

“**Haemorrhagic stroke**” refers to a stroke resulting from any rupture in any of the vasculature of the brain.

- Examples of acute neurological disorders that include stroke or involve aetiology or
- 15 symptoms such as those observed with stroke are listed above, and include, without limitation, cerebral ischemia or infarction (including embolic occlusion and thrombotic occlusion), reperfusion following acute ischemia, perinatal hypoxic-ischemic injury, cardiac arrest, as well as intracranial haemorrhages of any type (such as, *e.g.*, epidural, subdural, subarachnoid and intracerebral).

- 20 “**Subject**”, as used herein, refers to an individual to be diagnosed or treated according to the methods of the present invention. Subjects include, but are not limited to, mammals (*e.g.*, murines, simians, equines, bovines, porcines, canines, felines, and the like), preferably to primates, and most preferably to humans. In the context of the invention, the term “**patient**” generally refers to an individual seeking or having sought a diagnosis
- 25 or treatment according to the methods of the present invention.

“**Treating**” or “**treatment**”, as used herein, refers to alleviating a specified condition (such as a stroke), eliminating or reducing the symptoms of a condition (such as a stroke), slowing or eliminating the progression of a condition (such as a stroke), and preventing or delaying the initial occurrence of a condition (such as a stroke) in a subject, or

preventing or delaying the reoccurrence of a condition (such as a stroke) in a previously afflicted subject.

“**Diagnosing**” or “**diagnosis**”, as used herein, refers to assessing the development or progression of a condition (such as a stroke). As is known to a person skilled in the art, the assessment can be accurately performed for a statistically significant subject, although it is intended to be accurate for 100% of the subjects to be diagnosed. Statistical significance can be easily determined by a person skilled in the art using methods widely known in the art, *e.g.*, confidence interval determination, *p*-value determination, *t*-test, Mann-Whitney test, and the like. Preferred confidence intervals are 90% or higher, 95% or higher, 97% or higher, 98% or higher, and 99%. A preferred *p*-value is 0.1, 0.05, 0.01, 0.005 or 0.0001. Preferably, diagnosis results according to the present invention will be accurate for 60% or more, 70% or more, 80% or more, or 90% or more of a group of subjects.

“**Prognosing**” or “**prognosis**”, as used herein, refers to predicating the outcome for a subject with a condition (such as a stroke), after a particular treatment or intervention.

“**Blood sample**”, as used herein, means any blood sample derived or obtained from a subject. Collections of blood samples can be performed by methods well known to those skilled in the art. In some embodiments, the blood sample is a whole blood sample, a serum sample or a plasma sample.

“**Biomarker**”, as used herein, refers to a set of products of gene expression (*e.g.*, mRNA and/or protein) that is associated with brain tissue or neural cells injury, and which can be correlated with stroke, but is preferably not correlated with other types of injury. Such specific biomarkers of stroke identified in the blood by the Inventors include *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *GOS2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*. These specific biomarkers are described in detail hereinafter.

In the present specification, the name of each of the genes of interest refers to the internationally recognised name of the corresponding gene, as found in internationally recognised gene sequences and protein sequences databases, in particular in the database from the HUGO Gene Nomenclature Committee, that is available at the following Internet address: <http://www.gene.ucl.ac.uk/nomenclature/index.html>.

In the present specification, the name of each of the various biomarkers of interest may also refer to the internationally recognised name of the corresponding gene, as found in the internationally recognised gene sequences and protein sequences databases ENTRE ID, Genbank, TrEMBL or ENSEMBL. Through these internationally recognised
5 sequence databases, the nucleic acid sequences corresponding to each of the gene of interest described herein may be retrieved by the one skilled in the art.

“**PTGS2**”, as used herein, has its general meaning in the art and refers to prostaglandin-endoperoxide synthase 2 gene (Gene ID: 5743). An exemplary human amino acid sequence encoded by the “**PTGS2**” gene is represented by the NCBI reference sequence
10 NP_000954.1 (SEQ ID NO: 1). An exemplary human mRNA sequence of the “**PTGS2**” gene is represented by the NCBI reference sequence NM_000963.4 (SEQ ID NO: 2), with the coding sequence (CDS) ranging from residue 134 to residue 1948 of SEQ ID NO: 2.

“**HMOXI**”, as used herein, has its general meaning in the art and refers to heme oxygenase 1 gene (Gene ID: 3162). An exemplary human amino acid sequence encoded
15 by the “**HMOXI**” gene is represented by the NCBI reference sequence NP_002124.1 (SEQ ID NO: 3). An exemplary human mRNA sequence of the “**HMOXI**” gene is represented by the NCBI reference sequence NM_002133.3 (SEQ ID NO: 4), with the coding sequence (CDS) ranging from residue 79 to residue 945 of SEQ ID NO: 4.

“**LDLR**”, as used herein, has its general meaning in the art and refers to low density lipoprotein receptor gene (Gene ID: 3949). An exemplary human amino acid sequence
20 encoded by the “**LDLR**” gene is represented by the NCBI reference sequence NP_000518.1 (SEQ ID NO: 5). An exemplary human mRNA sequence of the “**LDLR**” gene is represented by the NCBI reference sequence NM_000527.4 (SEQ ID NO: 6), with the coding sequence (CDS) ranging from residue 188 to residue 2770 of SEQ ID NO: 6.

25 “**HSPA1B**”, as used herein, has its general meaning in the art and refers to heat shock protein family A (Hsp70) member 1B gene (Gene ID: 3304). An exemplary human amino acid sequence encoded by the “**HSPA1B**” gene is represented by the NCBI reference sequence NP_005337.2 (SEQ ID NO: 7). An exemplary human mRNA sequence of the “**HSPA1B**” gene is represented by the NCBI reference sequence NM_005346.5 (SEQ ID

NO: 8), with the coding sequence (CDS) ranging from residue 214 to residue 2139 of SEQ ID NO: 8.

“**G0S2**”, as used herein, has its general meaning in the art and refers to G0/G1 switch 2 gene (Gene ID: 50486). An exemplary human amino acid sequence encoded by the
5 “**G0S2**” gene is represented by the NCBI reference sequence NP_056529.1 (SEQ ID NO: 9). An exemplary human mRNA sequence of the “**G0S2**” gene is represented by the NCBI reference sequence NM_015714.4 (SEQ ID NO: 10), with the coding sequence (CDS) ranging from residue 171 to residue 482 of SEQ ID NO: 10.

“**BAG3**”, as used herein, has its general meaning in the art and refers to BCL2 associated
10 athanogene 3 gene (Gene ID: 9531). An exemplary human amino acid sequence encoded by the “**BAG3**” gene is represented by the NCBI reference sequence NP_004272.2 (SEQ ID NO: 11). An exemplary human mRNA sequence of the “**BAG3**” gene is represented by the NCBI reference sequence NM_004281.3 (SEQ ID NO: 12), with the coding sequence (CDS) ranging from residue 307 to residue 2034 of SEQ ID NO: 12.

15 “**TM4SFT**”, as used herein, has its general meaning in the art and refers to transmembrane 4 L six family member 1 gene (Gene ID: 4071). An exemplary human amino acid sequence encoded by the “**TM4SFT**” gene is represented by the NCBI reference sequence XP_016861874.1 (SEQ ID NO: 13). An exemplary human mRNA sequence of the “**TM4SFT**” gene is represented by the NCBI reference sequence XM_017006385.2 (SEQ
20 ID NO: 14), with the coding sequence (CDS) ranging from residue 235 to residue 954 of SEQ ID NO: 14.

“**DUSPI**”, as used herein, has its general meaning in the art and refers to dual specificity phosphatase 1 gene (Gene ID: 1843). An exemplary human amino acid sequence encoded by the “**DUSPI**” gene is represented by the NCBI reference sequence NP_004408.1 (SEQ
25 ID NO: 15). An exemplary human mRNA sequence of the “**DUSPI**” gene is represented by the NCBI reference sequence NM_004417.4 (SEQ ID NO: 16), with the coding sequence (CDS) ranging from residue 244 to residue 1347 of SEQ ID NO: 16.

“**ADM**”, as used herein, has its general meaning in the art and refers to adrenomedullin gene (Gene ID: 133). An exemplary human amino acid sequence encoded by the “**ADM**”
30 gene is represented by the NCBI reference sequence NP_001115.1 (SEQ ID NO: 17). An

exemplary human mRNA sequence of the “*ADM*” gene is represented by the NCBI reference sequence NM_001124.3 (SEQ ID NO: 18), with the coding sequence (CDS) ranging from residue 179 to residue 736 of SEQ ID NO: 18.

5 DETAILED DESCRIPTION

The present invention relates to a signature of stroke, wherein said signature comprises biomarkers whose expression levels are specific or indicative of a stroke. Such biomarkers will be hereinafter referred to as a “**stroke biomarker**”.

In one embodiment, the signature of the invention is specific or indicative of stroke. In one embodiment, the signature of the invention is specific or indicative of ischemic stroke, mini-stroke (also known as transient ischemic attack, TIA) and/or haemorrhagic stroke (in particular, haemorrhagic stroke caused by intracerebral haemorrhage). In one embodiment, the signature of the invention is specific or indicative of ischemic stroke. In one embodiment, the signature of the invention is specific or indicative of transient ischemic attack. In one embodiment, the signature of the invention is specific or indicative of haemorrhagic stroke (in particular, haemorrhagic stroke caused by intracerebral haemorrhage).

In one embodiment, the signature of the invention comprises or consists of at least one stroke biomarker. In one embodiment, the signature of the invention comprises or consists of at least two stroke biomarkers. In one embodiment, the signature of the invention comprises or consists of at least three stroke biomarkers. In one embodiment, the signature of the invention comprises or consists of at least four stroke biomarkers. In one embodiment, the signature of the invention comprises or consists of at least five stroke biomarkers. In one embodiment, the signature of the invention comprises or consists of at least six stroke biomarkers. In one embodiment, the signature of the invention comprises or consists of at least seven stroke biomarkers. In one embodiment, the signature of the invention comprises or consists of at least eight stroke biomarkers. In one embodiment, the signature of the invention comprises or consists of at least nine stroke biomarkers.

In one embodiment, stroke biomarkers are selected from the group comprising or consisting of *HSPA1B*, *NPAS4*, *DNAJB1*, *ATF3*, *HSPB1*, *RRAD*, *NR4A1*, *CYR61*, *C-FOS*, *GADD45G*, *RGS1*, *ARC*, *EGR4*, *PTGS2*, *RGS2*, *CCL3*, *BAG3*, *EGR2*, *HSPA4L*, *ADM*, *TM4SF1*, *EGR1*, *DUSP1*, *BTG2*, *LOC715456*, *HMOX1*, *LDLR*, *DNAJA4*, *MCL1*, *HSPA6*,
5 *GADD45B*, *IL6*, *ADFP*, *HES4*, *DUSP5*, *GEM* and *G0S2*.

In one embodiment, stroke biomarkers are selected from the group comprising or consisting of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*.

In one embodiment, stroke biomarkers are selected from the group comprising or
10 consisting of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3* and *TM4SF1*.

In one embodiment, the signature of the invention comprises or consists of at least one stroke biomarker selected from the group comprising or consisting of *HSPA1B*, *NPAS4*, *DNAJB1*, *ATF3*, *HSPB1*, *RRAD*, *NR4A1*, *CYR61*, *C-FOS*, *GADD45G*, *RGS1*, *ARC*, *EGR4*, *PTGS2*, *RGS2*, *CCL3*, *BAG3*, *EGR2*, *HSPA4L*, *ADM*, *TM4SF1*, *EGR1*, *DUSP1*,
15 *BTG2*, *LOC715456*, *HMOX1*, *LDLR*, *DNAJA4*, *MCL1*, *HSPA6*, *GADD45B*, *IL6*, *ADFP*, *HES4*, *DUSP5*, *GEM* and *G0S2*.

In one embodiment, the signature of the invention comprises or consists of at least one stroke biomarker selected from the group comprising or consisting of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*.

20 In one embodiment, the signature of the invention comprises or consists of at least one stroke biomarker selected from the group comprising or consisting of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3* and *TM4SF1*.

In one embodiment, the signature of the invention comprises or consists of at least two stroke biomarkers selected from the group comprising or consisting of *HSPA1B*, *NPAS4*,
25 *DNAJB1*, *ATF3*, *HSPB1*, *RRAD*, *NR4A1*, *CYR61*, *C-FOS*, *GADD45G*, *RGS1*, *ARC*, *EGR4*, *PTGS2*, *RGS2*, *CCL3*, *BAG3*, *EGR2*, *HSPA4L*, *ADM*, *TM4SF1*, *EGR1*, *DUSP1*, *BTG2*, *LOC715456*, *HMOX1*, *LDLR*, *DNAJA4*, *MCL1*, *HSPA6*, *GADD45B*, *IL6*, *ADFP*, *HES4*, *DUSP5*, *GEM* and *G0S2*.

In one embodiment, the signature of the invention comprises or consists of at least two stroke biomarkers selected from the group comprising or consisting of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*.

5 In one embodiment, the signature of the invention comprises or consists of at least two stroke biomarkers selected from the group comprising or consisting of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3* and *TM4SF1*.

In one embodiment, the signature of the invention comprises or consists of at least two stroke biomarkers selected from the group comprising or consisting of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*, with the proviso that the at least two biomarkers do not consist in *DUSP1* and *ADM*.

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In one embodiment, the signature of the invention comprises or consists of at least three stroke biomarkers selected from the group comprising or consisting of *HSPA1B*, *NPAS4*, *DNAJB1*, *ATF3*, *HSPB1*, *RRAD*, *NR4A1*, *CYR61*, *C-FOS*, *GADD45G*, *RGS1*, *ARC*, *EGR4*, *PTGS2*, *RGS2*, *CCL3*, *BAG3*, *EGR2*, *HSPA4L*, *ADM*, *TM4SF1*, *EGR1*, *DUSP1*, *BTG2*, *LOC715456*, *HMOX1*, *LDLR*, *DNAJA4*, *MCL1*, *HSPA6*, *GADD45B*, *IL6*, *ADFP*, *HES4*, *DUSP5*, *GEM* and *G0S2*.

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In one embodiment, the signature of the invention comprises or consists of at least three stroke biomarkers selected from the group comprising or consisting of, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*.

20 In one embodiment, the signature of the invention comprises or consists of at least three stroke biomarkers selected from the group comprising or consisting of, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3* and *TM4SF1*.

In one embodiment, the signature of the invention comprises or consists of at least four stroke biomarkers selected from the group comprising or consisting of, *HSPA1B*, *NPAS4*, *DNAJB1*, *ATF3*, *HSPB1*, *RRAD*, *NR4A1*, *CYR61*, *C-FOS*, *GADD45G*, *RGS1*, *ARC*, *EGR4*, *PTGS2*, *RGS2*, *CCL3*, *BAG3*, *EGR2*, *HSPA4L*, *ADM*, *TM4SF1*, *EGR1*, *DUSP1*, *BTG2*, *LOC715456*, *HMOX1*, *LDLR*, *DNAJA4*, *MCL1*, *HSPA6*, *GADD45B*, *IL6*, *ADFP*, *HES4*, *DUSP5*, *GEM* and *G0S2*.

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In one embodiment, the signature of the invention comprises or consists of at least four stroke biomarkers selected from the group comprising or consisting of, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*.

5 In one embodiment, the signature of the invention comprises or consists of at least four stroke biomarkers selected from the group comprising or consisting of, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3* and *TM4SF1*.

In one embodiment, the signature of the invention comprises or consists of at least five stroke biomarkers selected from the group comprising or consisting of, *HSPA1B*, *NPAS4*, *DNAJB1*, *ATF3*, *HSPB1*, *RRAD*, *NR4A1*, *CYR61*, *C-FOS*, *GADD45G*, *RGS1*, *ARC*,
10 *EGR4*, *PTGS2*, *RGS2*, *CCL3*, *BAG3*, *EGR2*, *HSPA4L*, *ADM*, *TM4SF1*, *EGR1*, *DUSP1*, *BTG2*, *LOC715456*, *HMOX1*, *LDLR*, *DNAJA4*, *MCL1*, *HSPA6*, *GADD45B*, *IL6*, *ADFP*, *HES4*, *DUSP5*, *GEM* and *G0S2*.

In one embodiment, the signature of the invention comprises or consists of at least five stroke biomarkers selected from the group comprising or consisting of, *PTGS2*, *HMOX1*,
15 *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*.

In one embodiment, the signature of the invention comprises or consists of at least five stroke biomarkers selected from the group comprising or consisting of, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3* and *TM4SF1*.

In one embodiment, the signature of the invention comprises or consists of at least six stroke biomarkers selected from the group comprising or consisting of, *HSPA1B*, *NPAS4*, *DNAJB1*, *ATF3*, *HSPB1*, *RRAD*, *NR4A1*, *CYR61*, *C-FOS*, *GADD45G*, *RGS1*, *ARC*,
20 *EGR4*, *PTGS2*, *RGS2*, *CCL3*, *BAG3*, *EGR2*, *HSPA4L*, *ADM*, *TM4SF1*, *EGR1*, *DUSP1*, *BTG2*, *LOC715456*, *HMOX1*, *LDLR*, *DNAJA4*, *MCL1*, *HSPA6*, *GADD45B*, *IL6*, *ADFP*, *HES4*, *DUSP5*, *GEM* and *G0S2*.

25 In one embodiment, the signature of the invention comprises or consists of at least six stroke biomarkers selected from the group comprising or consisting of, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*.

In one embodiment, the signature of the invention comprises or consists of at least six stroke biomarkers selected from the group comprising or consisting of, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3* and *TM4SF1*.

5 In one embodiment, the signature of the invention comprises or consists of at least seven stroke biomarkers selected from the group comprising or consisting of, *HSPA1B*, *NPAS4*, *DNAJB1*, *ATF3*, *HSPB1*, *RRAD*, *NR4A1*, *CYR61*, *C-FOS*, *GADD45G*, *RGS1*, *ARC*, *EGR4*, *PTGS2*, *RGS2*, *CCL3*, *BAG3*, *EGR2*, *HSPA4L*, *ADM*, *TM4SF1*, *EGR1*, *DUSP1*, *BTG2*, *LOC715456*, *HMOX1*, *LDLR*, *DNAJA4*, *MCL1*, *HSPA6*, *GADD45B*, *IL6*, *ADFP*, *HES4*, *DUSP5*, *GEM* and *G0S2*.

10 In one embodiment, the signature of the invention comprises or consists of at least seven stroke biomarkers selected from the group comprising or consisting of, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*.

In one embodiment, the signature of the invention comprises or consists of at least seven stroke biomarkers selected from the group comprising or consisting of, *PTGS2*, *HMOX1*,
15 *LDLR*, *HSPA1B*, *G0S2*, *BAG3* and *TM4SF1*.

In one embodiment, the signature of the invention comprises or consists of at least eight stroke biomarkers selected from the group comprising or consisting of, *HSPA1B*, *NPAS4*, *DNAJB1*, *ATF3*, *HSPB1*, *RRAD*, *NR4A1*, *CYR61*, *C-FOS*, *GADD45G*, *RGS1*, *ARC*, *EGR4*, *PTGS2*, *RGS2*, *CCL3*, *BAG3*, *EGR2*, *HSPA4L*, *ADM*, *TM4SF1*, *EGR1*, *DUSP1*,
20 *BTG2*, *LOC715456*, *HMOX1*, *LDLR*, *DNAJA4*, *MCL1*, *HSPA6*, *GADD45B*, *IL6*, *ADFP*, *HES4*, *DUSP5*, *GEM* and *G0S2*.

In one embodiment, the signature of the invention comprises or consists of at least eight stroke biomarkers selected from the group comprising or consisting of, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*.

25 In one embodiment, the signature of the invention comprises or consists of at least nine stroke biomarkers selected from the group comprising or consisting of, *HSPA1B*, *NPAS4*, *DNAJB1*, *ATF3*, *HSPB1*, *RRAD*, *NR4A1*, *CYR61*, *C-FOS*, *GADD45G*, *RGS1*, *ARC*, *EGR4*, *PTGS2*, *RGS2*, *CCL3*, *BAG3*, *EGR2*, *HSPA4L*, *ADM*, *TM4SF1*, *EGR1*, *DUSP1*,

BTG2, LOC715456, HMOX1, LDLR, DNAJA4, MCL1, HSPA6, GADD45B, IL6, ADFP, HES4, DUSP5, GEM and G0S2.

In one embodiment, the signature of the invention comprises or consists of at least nine stroke biomarkers selected from the group comprising or consisting of, *PTGS2, HMOX1,*
5 *LDLR, HSPA1B, G0S2, BAG3, TM4SF1, DUSP1* and *ADM.*

The present invention thus also relates to a signature of stroke comprising or consisting of one or several biomarker(s) whose expression level(s) is/are different between a subject affected with a stroke and a substantially healthy subject.

In one embodiment, the signature of the invention is specific or indicative of stroke if the
10 expression level of at least one stroke biomarker selected from the group comprising or consisting of, *PTGS2, HMOX1, LDLR, HSPA1B, G0S2, BAG3, TM4SF1, DUSP1* and *ADM* is upregulated, as compared to a reference signature.

In one embodiment, the signature of the invention is specific or indicative of stroke if the expression level of at least one stroke biomarker selected from the group comprising or
15 consisting of, *PTGS2, HMOX1, LDLR, HSPA1B, G0S2, BAG3* and *TM4SF1* is upregulated, as compared to a reference signature.

In one embodiment, the signature of the invention is specific or indicative of stroke if the expression level of at least two stroke biomarkers selected from the group comprising or consisting of, *PTGS2, HMOX1, LDLR, HSPA1B, G0S2, BAG3, TM4SF1, DUSP1* and
20 *ADM* is upregulated, as compared to a reference signature; preferably with the proviso that the at least two biomarkers do not consist in *DUSP1* and *ADM.*

In one embodiment, the signature of the invention is specific or indicative of stroke if the expression level of at least two stroke biomarkers selected from the group comprising or consisting of, *PTGS2, HMOX1, LDLR, HSPA1B, G0S2, BAG3* and *TM4SF1* is
25 upregulated, as compared to a reference signature.

In one embodiment, the signature of the invention is specific or indicative of stroke if the expression level of at least three stroke biomarkers selected from the group comprising

or consisting of, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM* is upregulated, as compared to a reference signature.

In one embodiment, the signature of the invention is specific or indicative of stroke if the expression level of at least three stroke biomarkers selected from the group comprising
5 or consisting of, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3* and *TM4SF1* is upregulated, as compared to a reference signature.

In one embodiment, the signature of the invention is specific or indicative of stroke if the expression level of at least four stroke biomarkers selected from the group comprising or consisting of, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and
10 *ADM* is upregulated, as compared to a reference signature.

In one embodiment, the signature of the invention is specific or indicative of stroke if the expression level of at least four stroke biomarkers selected from the group comprising or consisting of, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3* and *TM4SF1* is upregulated, as compared to a reference signature.

15 In one embodiment, the signature of the invention is specific or indicative of stroke if the expression level of at least five stroke biomarkers selected from the group comprising or consisting of, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM* is upregulated, as compared to a reference signature.

In one embodiment, the signature of the invention is specific or indicative of stroke if the expression level of at least five stroke biomarkers selected from the group comprising or
20 consisting of, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3* and *TM4SF1* is upregulated, as compared to a reference signature.

In one embodiment, the signature of the invention is specific or indicative of stroke if the expression level of at least six stroke biomarkers selected from the group comprising or
25 consisting of, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM* is upregulated, as compared to a reference signature.

In one embodiment, the signature of the invention is specific or indicative of stroke if the expression level of at least six stroke biomarkers selected from the group comprising or

consisting of, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3* and *TM4SF1* is upregulated, as compared to a reference signature.

In one embodiment, the signature of the invention is specific or indicative of stroke if the expression level of at least seven stroke biomarkers selected from the group comprising
5 or consisting of, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM* is upregulated, as compared to a reference signature.

In one embodiment, the signature of the invention is specific or indicative of stroke if the expression level of at least seven stroke biomarkers selected from the group comprising
10 or consisting of, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3* and *TM4SF1* is upregulated, as compared to a reference signature.

In one embodiment, the signature of the invention is specific or indicative of stroke if the expression level of at least eight stroke biomarkers selected from the group comprising
or consisting of, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM* is upregulated, as compared to a reference signature.

15 In one embodiment, the signature of the invention is specific or indicative of stroke if the expression level of at least nine stroke biomarkers selected from the group comprising
or consisting of, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM* is upregulated, as compared to a reference signature.

20 In one embodiment, the expression level of the stroke biomarkers of the invention in a sample from a subject, preferably a bodily fluid sample, more preferably a blood sample, can be determined using standard protocols known in the art.

In one embodiment, the expression level of the stroke biomarkers of the invention corresponds to their transcription levels (*i.e.*, the expression of the mRNA) or to their translation levels (*i.e.*, expression of the protein).

25 In one embodiment, the expression level of the stroke biomarkers is assessed at the protein level, *i.e.*, at the translation level, in a sample, preferably a bodily fluid sample, more preferably a blood sample, from a subject. In this embodiment, the signature according to the present invention may be referred to as a proteomic signature.

Methods for determining a protein level in a sample are well known in the art. Examples of such methods include, but are not limited to, immunohistochemistry, multiplex methods (Luminex), western blot, enzyme-linked immunosorbent assay (ELISA), sandwich ELISA, fluorescent-linked immunosorbent assay (FLISA), enzyme immunoassay (EIA), radioimmunoassay (RIA), mass spectrometry (MS), a microarray, and the like, or any combination thereof.

Mass spectrometry (MS) can be used to resolve different forms of a protein because the different forms typically have different masses that can be resolved by mass spectrometry. Accordingly, if one form of a polypeptide or protein is a better biomarker for a disease than another form of the biomarker, mass spectrometry can be used to specifically detect and measure the useful form. MS can include time-of-flight (TOF) MS (*e.g.*, matrix-assisted laser desorption/ionization (MALDI)-TOF MS), surface-enhanced laser desorption/ionization (MELDI) MS, electrospray ionization MS, or Fourier transform ion cyclotron resonance (FT-ICR) MS.

Immunoassays typically comprise contacting the sample with a binding partner capable of selectively interacting with the biomarker in the sample. In some embodiments, the binding partners are antibodies, such as, *e.g.*, monoclonal antibodies or even aptamers. For example, the binding may be detected through use of a competitive immunoassay, a non-competitive assay system using techniques such as western blots, a radioimmunoassay, an ELISA, a “sandwich” immunoassay, an immunoprecipitation assay, a precipitin reaction, a gel diffusion precipitin reaction, an immunodiffusion assay, an agglutination assay, a complement fixation assay, an immunoradiometric assay, a fluorescent immunoassay, a protein A immunoassay, an immunoprecipitation assay, an immunohistochemical assay, a competition or sandwich ELISA, a radioimmunoassay, a Western blot assay, an immunohistological assay, an immunocytochemical assay, a dot blot assay, a fluorescence polarization assay, a scintillation proximity assay, a homogeneous time resolved fluorescence assay, a IAsys analysis, and a BIAcore analysis. The aforementioned assays generally involve the binding of the partner (*i.e.*, antibody or aptamer) to a solid support. Solid supports which can be used in the practice of the invention include substrates such as nitrocellulose (*e.g.*, in membrane or microtiter well

form), polyvinylchloride (*e.g.*, sheets or microtiter wells), polystyrene latex (*e.g.*, beads or microtiter plates), polyvinylidene fluoride, diazotized paper, nylon membranes, activated beads, magnetically responsive beads, and the like.

5 A multiplex assay can include a phage display, an antibody profiling, or an assay using a Luminex platform. A microarray for analysing a profile of polypeptides can include analytical microarrays, functional protein microarrays, or reverse phase protein microarrays. In some cases, a profile of polypeptides or proteins can be measured by a proteomic scan (*e.g.* a whole proteomic scan) using a proteomic microarray.

10 In one embodiment, the expression level of the stroke biomarkers is assessed at the nucleic acid level (*i.e.*, RNA), *i.e.*, at the transcription level, in a sample, preferably a bodily fluid sample, more preferably a blood sample, from a subject. In this embodiment, the signature according to the present invention may be referred to as a transcriptomic signature.

Methods for assessing the transcription level of a biomarker are well known in the art. 15 Examples of such methods include, but are not limited to, polymerase chain reaction (PCR), RT-PCR, RT-qPCR, Northern Blot, hybridization techniques such as, for example, use of microarrays, and combination thereof including but not limited to, hybridization of amplicons obtained by RT-PCR, sequencing such as, for example, next-generation DNA sequencing (NGS) or RNA-seq (also known as “whole transcriptome 20 shotgun sequencing”) and the like, or any combination thereof.

Conventional methods typically involve polymerase chain reaction (PCR). For instance, US Patents US4,683,202, US4,683,195, US4,800,159 and US4,965,188 disclose conventional PCR techniques. PCR typically employs two oligonucleotide primers that bind to a selected target nucleic acid sequence. Primers useful in the present invention 25 include oligonucleotides capable of acting as a point of initiation of nucleic acid synthesis within the target nucleic acid sequence. A primer can be purified from a restriction digest by conventional methods, or it can be produced synthetically. PCR involves the use of a thermostable polymerase. The term “thermostable polymerase” refers to a polymerase enzyme that is heat stable, *i.e.*, the enzyme catalyses the formation of primer extension

products complementary to a template and does not irreversibly denature when subjected to the elevated temperatures for the time necessary to effect denaturation of double-stranded template nucleic acids. Thermostable polymerases have been isolated from *Thermus flavus*, *T. ruber*, *T. thermophilus*, *T. aquaticus*, *T. lacteus*, *T. rubens*, *Bacillus*
5 *stearothermophilus*, and *Methanothermus fervidus*. Nonetheless, polymerases that are not thermostable also can be employed in PCR assays provided the enzyme is replenished. Typically, the polymerase is a Taq polymerase (*i.e.*, *Thermus aquaticus* polymerase).

Quantitative PCR (qPCR) is typically carried out in a thermal cycler with the capacity to illuminate each sample with a beam of light of a specified wavelength and detect the
10 fluorescence emitted by the excited fluorophore. The thermal cycler is also able to rapidly heat and chill samples, thereby taking advantage of the physicochemical properties of the nucleic acids and thermal polymerase. In order to detect and measure the amount of amplicon (*i.e.*, amplified target nucleic acid sequence) in the sample, a measurable signal has to be generated, which is proportional to the amount of amplified product. All current
15 detection systems use fluorescent technologies. Some of them are non-specific techniques, and consequently only allow the detection of one target at a time.

Alternatively, specific detection chemistries can distinguish between non-specific amplification and target amplification. These specific techniques can be used to multiplex the assay, *i.e.*, detecting several different targets in the same assay. For example, SYBR®
20 Green I probes, High Resolution Melting probes, TaqMan® probes, LNA® probes and Molecular Beacon probes can be suitable. TaqMan® probes are the most widely used type of probes. They were developed by Roche (Basel, Switzerland) and ABI (Foster City, USA) from an assay that originally used a radio-labelled probe (Holland *et al.*, 1991. *Proc Natl Acad Sci U S A.* **88**(16):7276-80), which consisted of a single-stranded probe
25 sequence that was complementary to one of the strands of the amplicon. A fluorophore is attached to the 5' end of the probe and a quencher to the 3' end. The fluorophore is excited by the machine and passes its energy, via FRET (Fluorescence Resonance Energy Transfer) to the quencher. Traditionally, the FRET pair has been conjugated to FAM as the fluorophore and TAMRA as the quencher. In a well-designed probe, FAM does not
30 fluoresce as it passes its energy onto TAMRA. As TAMRA fluorescence is detected at a

different wavelength to FAM, the background level of FAM is low. The probe binds to the amplicon during each annealing step of the PCR. When the Taq polymerase extends from the primer which is bound to the amplicon, it displaces the 5' end of the probe, which is then degraded by the 5'-3' exonuclease activity of the Taq polymerase. Cleavage continues until the remaining probe melts off the amplicon. This process releases the fluorophore and quencher into solution, spatially separating them (compared to when they were held together by the probe). This leads to an irreversible increase in fluorescence from the FAM and a decrease in the TAMRA.

In some embodiments, the expression level of the stroke biomarkers at the nucleic acid level (*i.e.*, RNA) is determined by RNA-seq. As used, the term "RNA-seq" or "transcriptome sequencing" refers to sequencing performed on RNA (or cDNA) instead of DNA, where typically, the primary goal is to measure expression levels, detect fusion transcripts, alternative splicing, and other genomic alterations that can be better assessed from RNA. RNA-seq typically includes whole transcriptome sequencing. As used herein, the term "whole transcriptome sequencing" refers to the use of high throughput sequencing technologies to sequence the entire transcriptome in order to get information about a sample's RNA content. Whole transcriptome sequencing can be done with a variety of platforms for example, the Genome Analyzer (Illumina, Inc., San Diego, Calif.) and the SOLiD™ Sequencing System (Life Technologies, Carlsbad, Calif.). However, any platform useful for whole transcriptome sequencing may be used. Typically, the RNA is extracted, and ribosomal RNA may be deleted as described in US Patent US9,005,891. cDNA sequencing libraries may be prepared, that are directional and single or paired-end using commercially available kits such as the ScriptSeq™ M mRNA-Seq Library Preparation Kit (Epicenter Biotechnologies, Madison, Wis.). The libraries may also be barcoded for multiplex sequencing using commercially available barcode primers such as the RNA-Seq Barcode Primers from Epicenter Biotechnologies (Madison, Wis.). PCR is then carried out to generate the second strand of cDNA to incorporate the barcodes and to amplify the libraries. After the libraries are quantified, the sequencing libraries may be sequenced. Nucleic acid sequencing technologies are suitable methods for expression analysis. The principle underlying these methods is that the number of times a DNA sequence is detected in a sample is directly related to the relative RNA levels

corresponding to that sequence. These methods are sometimes referred to by the term “Digital Gene Expression” or DOE, to reflect the discrete numeric property of the resulting data. Early methods applying this principle were Serial Analysis of Gene Expression (SAGE) and Massively Parallel Signature Sequencing (MPSS). See, *e.g.*,
5 Brenner *et al.* (2000. *Nat Biotechnol.* **18**(6):630-634). Typically, RNA-seq uses Next Generation Sequencing (or NGS). As used herein, the term “Next Generation Sequencing” or “NGS” refers to a relatively new sequencing technique as compared to the traditional Sanger sequencing technique. For a review, see Shendure *et al.* (2008. *Nat Biotechnol.* **26**(10):1135-45), which is hereby incorporated by reference into this
10 disclosure. For the purpose of this disclosure, NGS may include cyclic array sequencing, microelectrophoretic sequencing, sequencing by hybridization, among others. By way of example, in a typical NGS using cyclic-array methods, genomic DNA or cDNA library is first prepared, and common adaptors may then be ligated to the fragmented genomic DNA or cDNA. Different protocols may be used to generate jumping libraries of mate-
15 paired tags with controllable distance distribution. An array of millions of spatially immobilized PCR colonies or “polonies” is generated with each polonies consisting of many copies of a single shotgun library fragment. Because the polonies are tethered to a planar array, a single microliter-scale reagent volume can be applied to manipulate the array features in parallel, *e.g.*, for primer hybridization or for enzymatic extension
20 reactions. Imaging-based detection of fluorescent labels incorporated with each extension may be used to acquire sequencing data on all features in parallel. Successive iterations of enzymatic interrogation and imaging may also be used to build up a contiguous sequencing read for each array feature.

In one embodiment, the decision as to whether the expression level of a certain stroke
25 biomarker in a specific sample, preferably a bodily fluid sample, more preferably a blood sample, is upregulated is taken in comparison to a reference signature and/or a predetermined reference value.

In one embodiment, a reference signature comprises or consists of predetermined reference values for each of the stroke biomarkers of interest, preferably of at least 1, 2,
30 3, 4, 5, 6, 7, 8, 9 or more stroke biomarkers selected from the group comprising or

consisting of *HSPA1B*, *NPAS4*, *DNAJB1*, *ATF3*, *HSPB1*, *RRAD*, *NR4A1*, *CYR61*, *C-FOS*, *GADD45G*, *RGS1*, *ARC*, *EGR4*, *PTGS2*, *RGS2*, *CCL3*, *BAG3*, *EGR2*, *HSPA4L*, *ADM*, *TM4SF1*, *EGR1*, *DUSP1*, *BTG2*, *LOC715456*, *HMOX1*, *LDLR*, *DNAJA4*, *MCL1*, *HSPA6*, *GADD45B*, *IL6*, *ADFP*, *HES4*, *DUSP5*, *GEM* and *G0S2*,

- 5 preferably selected from the group comprising or consisting of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*,
preferably with the proviso that the at least one or two stroke biomarkers do not consist in *DUSP1* and/or *ADM*.

Typically, a reference signature may be either implemented in the software or an overall
10 median or other arithmetic mean across measurements may be built.

In one embodiment, the reference signature is derived from a previous measurement of the expression levels of stroke biomarkers according to the invention, in a reference sample derived from the same subject, such as, for example, the expression levels of stroke biomarkers measured one month before the subsequent measurement of the
15 expression levels of stroke biomarkers according to the invention, preferably six months before, more preferably one year before or more; or such as, for example, the expression levels of stroke biomarkers measured before starting a therapy.

In one embodiment, the reference signature is derived from the measurement of the expression levels of stroke biomarkers according to the invention, in a reference
20 population.

In one embodiment, the reference signature can be relative to a signature derived from population studies, including without limitation, such subjects having similar age range, subjects in the same or similar ethnic group, similar cancer history and the like.

In one embodiment, the reference signature is derived from the measurement of the
25 expression levels of stroke biomarkers according to the invention, in a control sample derived from one or more substantially healthy subject(s). As used herein, a “**substantially healthy subject**” has not been previously diagnosed or identified as having or suffering from a stroke.

In one embodiment, the reference population comprises substantially healthy subjects, preferably at least 50, more preferably at least 100, more preferably at least 200 and even more preferably at least 500 substantially healthy subjects.

By implying a multitude of samples from the reference population, it is conceivable to
5 calculate a median and/or mean expression level for each stroke biomarker respectively. In relation to these results, a stroke biomarker can be monitored as differential expressed. In one embodiment, the reference signature corresponds to the mean expression levels of the stroke biomarkers of the signature of the invention, measured in the reference population. In one embodiment, the reference signature corresponds to the median
10 expression levels of the stroke biomarkers of the signature of the invention, measured in the reference population.

In one embodiment, the reference signature is constructed using algorithms and other methods of statistical and structural classification. Samples from the reference population are used to compute a mean profile (*i.e.*, a reference signature) of the at least one stroke
15 biomarker, preferably at least 2, 3, 4, 5, 6, 7, 8 or 9 stroke biomarkers according to the invention.

In one embodiment, a stroke biomarker is considered as differentially expressed between a subject to be diagnosed as being affected or not with a stroke and a substantially healthy subject if the expression fold change is at least greater than about |1.1|, preferably at least
20 greater than about |1.2|, about |1.3|, about |1.4|, about |1.5|, about |1.6|, about |1.7|, about |1.8|, about |1.9|, about |2.0|, about |2.1|, about |2.2|, about |2.3|, about |2.4|, about |2.5|, about |3.0|, about |4.0|, about |5.0| or more.

In one embodiment, a stroke biomarker is considered as differentially expressed between a subject to be diagnosed as being affected or not with a stroke and a substantially healthy
25 subject if the expression fold change is at least greater than about 1.1, preferably at least greater than about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9, about 2.0, about 2.1, about 2.2, about 2.3, about 2.4, about 2.5, about 3.0, about 4.0, about 5.0 or more.

In one embodiment, a stroke biomarker is considered as differentially expressed between a subject to be diagnosed as being affected or not with a stroke and a substantially healthy subject if the log₂ expression fold change is at least greater than about |0.1|, preferably at least greater than about |0.2|, about |0.3|, about |0.4|, about |0.5|, about |0.6|, about |0.7|, about |0.8|, about |0.9|, about |1.0|, about |1.1|, about |1.2|, about |1.3|, about |1.4|, about |1.5|, about |1.6|, about |1.7|, about |1.8|, about |1.9|, about |2.0|, about |2.1|, about |2.2|, about |2.3| or more.

In one embodiment, a stroke biomarker is considered as differentially expressed between a subject to be diagnosed as being affected or not with a stroke and a substantially healthy subject if the log₂ expression fold change is at least greater than about 0.1, preferably at least greater than about 0.2, about 0.3, about 0.4, about 0.5, about 0.6, about 0.7, about 0.8, about 0.9, about 1.0, about 1.1, about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9, about 2.0, about 2.1, about 2.2, about 2.3 or more.

In one embodiment, a stroke biomarker is considered as differentially expressed between a subject to be diagnosed as being affected or not with a stroke and a substantially healthy subject if the expression level of said biomarker is at least about 0.5, about 1, about 1.5, about 2, about 2.5, about 3, about 3.5, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 15, about 20, about 30, about 40, about 50, or about 100 times higher than the expression level of the same biomarker determined in the substantially healthy subject, preferably in the reference population.

In one embodiment, when the expression level of more than one stroke biomarker is determined in a sample, preferably a bodily fluid sample, more preferably a blood sample, obtained from the subject to be diagnosed as being affected or not with a stroke, a score, which is a composite score of said expression levels, may be calculated and compared with a predetermined reference value. In one embodiment, a score higher than the predetermined reference value indicates that the subject has or has had a stroke.

Typically, the predetermined reference value is a threshold value or a cut-off value. Typically, a “threshold value” or “cut-off value” can be determined experimentally, empirically, or theoretically. A threshold value can also be arbitrarily selected based upon

the existing experimental and/or clinical conditions, as would be recognized by a person of ordinary skilled in the art. For example, retrospective measurement in properly banked historical subject samples may be used in establishing the predetermined reference value. The threshold value has to be determined in order to obtain the optimal sensitivity and specificity according to the function of the test and the benefit/risk balance (clinical consequences of false positive and false negative). Typically, the optimal sensitivity and specificity (and so the threshold value) can be determined using a Receiver Operating Characteristic (ROC) curve based on experimental data. For example, after determining the expression level of the biomarker of the invention in a reference population, one can use algorithmic analysis for the statistic treatment of the expression levels determined in samples to be tested, and thus obtain a classification standard having significance for sample classification. The full name of ROC curve is receiver operator characteristic curve, which is also known as receiver operation characteristic curve. It is mainly used for clinical biochemical diagnostic tests. ROC curve is a comprehensive indicator that reflects the continuous variables of true positive rate (sensitivity) and false positive rate (1-specificity). It reveals the relationship between sensitivity and specificity with the image composition method. A series of different cut-off values (thresholds or critical values, boundary values between normal and abnormal results of diagnostic test) are set as continuous variables to calculate a series of sensitivity and specificity values. Then sensitivity is used as the vertical coordinate and specificity is used as the horizontal coordinate to draw a curve. The higher the area under the curve (AUC), the higher the accuracy of diagnosis. On the ROC curve, the point closest to the far upper left of the coordinate diagram is a critical point having both high sensitivity and high specificity values. The AUC value of the ROC curve is between 1.0 and 0.5. When $AUC > 0.5$, the diagnostic result gets better and better as AUC approaches 1. When $0.5 < AUC < 0.7$, the accuracy is low. When $0.7 < AUC < 0.9$, the accuracy is moderate. When $AUC > 0.9$, the accuracy is high. This algorithmic method is preferably done with a computer. Existing software or systems in the art may be used for the drawing of the ROC curve, such as: MedCalc 9.2.0.1 medical statistical software, SPSS 9.0, ROCPOWER.SAS, DESIGNROC.FOR, MULTIREADER POWER.SAS, CREATE-ROC.SAS, GB STAT VI0.0 (Dynamic Microsystems, Inc. Silver Spring, Md., USA), etc.

The present invention further relates to a method of diagnosing a stroke in a subject, using the signature of stroke of the invention.

In one embodiment, the method of diagnosing a stroke according to the present invention is particularly suitable for diagnosing an ischemic stroke, a mini-stroke (or transient
5 ischemic attack, TIA) and/or a haemorrhagic stroke (in particular, haemorrhagic stroke caused by intracerebral haemorrhage).

In one embodiment, the method of diagnosing a stroke according to the present invention is particularly suitable for distinguishing a stroke from a stroke mimic, which can exhibit some or all of the same symptoms as a stroke, except that it is not a stroke and it is not a
10 result of brain ischemia. Without wishing to be bound by a theory, because an elevated level of the biomarker is the result of stroke, a subject having a stroke mimic will not have an elevated level of the biomarker (in other words, because the signature of the invention is specific or indicative of a stroke, a subject having a stroke mimic will not exhibit the same signature).

15 Common stroke mimics include, but are not limited to, migraine, syncope, peripheral vestibular disturbance and BPPV (benign paroxysmal positional vertigo), seizure, functional manifestation /anxiety, transient global amnesia, Bell's palsy, peripheral nerve disease/dysfunction, postural hypotension, tumor, viral illness, cardiac arrhythmia, multiple sclerosis, drug related, hypoglycaemia, Parkinson's disease, retinal/ocular
20 pathology, spinal pathology, trigeminal neuralgia, urinary tract infection, delirium, motor neuron disease, subarachnoid haemorrhage, subdural hematoma and the like (see Nadarajan *et al.*, **2014**. *Pract Neurol*. **14**(1):23-31).

Accordingly, the method of diagnosing a stroke according to the present invention provides the advantage of allowing an early and accurate diagnosis of stroke, which can
25 benefit a subject suspected of having stroke in at least the following related aspects:

- (1) it can reduce the rate of misdiagnosis of stroke; and/or
- (2) it can limit the extent of tissue death by permitting early and proper treatment in a subject in need of treatment.

In one embodiment, the method of diagnosing a stroke according to the present invention comprises a step of providing a sample from the subject to be diagnosed as being affected or not with a stroke.

The term “**sample**”, as used herein, generally refers to any sample which may be tested
5 for expression levels of a biomarker, preferably of stroke markers according to the present invention.

In one embodiment, the sample is a bodily fluid sample. Examples of bodily fluids include, but are not limited to, blood, plasma, serum, lymph, ascetic fluid, cystic fluid, urine, bile, mucus, serous fluid, sebum, nipple exudate, synovial fluid, bronchoalveolar
10 lavage fluid, sputum, amniotic fluid, peritoneal fluid, cerebrospinal fluid, pleural fluid, pericardial fluid, semen, saliva, tears, rheum, sweat and alveolar macrophages.

In one embodiment, the sample is a blood sample. The term “**blood sample**”, as used herein, encompasses whole blood samples, serum samples and plasma samples.

In one embodiment, the sample is a whole blood, plasma or serum sample.

15 In one embodiment, the sample is not a body tissue sample. Examples of body tissues include, but are not limited to, brain, muscle, nerve, heart, lung, liver, pancreas, spleen, thymus, oesophagus, stomach, intestine, kidney, prostate, testis, ovary, hair, skin, bone, breast, uterus, bladder and spinal cord.

In one embodiment, the sample is not a brain tissue sample. Therefore, according to this
20 embodiment, the methods of the invention do not comprise a step of providing a brain tissue sample from the subject.

In one embodiment, the sample is not a biopsy sample. In one embodiment, the sample is not a brain biopsy sample.

In one embodiment, the sample, preferably the bodily fluid sample, more preferably the
25 blood sample, was previously taken from the subject, *i.e.*, the method of diagnosing a stroke according to the present invention does not comprise a step of actively taking a

sample from the subject. Consequently, according to this embodiment, the method of the invention is a non-invasive method, *i.e.*, an *in vitro* method.

In one embodiment, the method of diagnosing a stroke according to the present invention comprises a step of determining the signature according to the present invention, in said
5 sample, preferably bodily fluid sample, more preferably blood sample, from the subject.

Means and methods for determining the signature according to the present invention are detailed hereinabove.

In one embodiment, the step of determining the signature comprises a substep of measuring the expression level of at least one stroke biomarker, preferably at least 2, 3,
10 4, 5, 6, 7, 8 or 9 stroke biomarkers according to the present invention.

In one embodiment, the expression level of the at least one stroke biomarker, preferably at least 2, 3, 4, 5, 6, 7, 8 or 9 stroke biomarkers, is measured using a DNA microarray, so that the expression levels of each of the stroke biomarkers of the signature of the invention are simultaneously measured.

15 In one embodiment, the expression level of the at least one stroke biomarker, preferably at least 2, 3, 4, 5, 6, 7, 8 or 9 stroke biomarkers, is measured using RNAseq.

In one embodiment, the expression level of the at least one stroke biomarker, preferably at least 2, 3, 4, 5, 6, 7, 8 or 9 stroke biomarkers, is measured using a CodeSet. Custom CodeSets for a given panel of markers (*e.g.*, for the stroke biomarkers disclosed herein)
20 are commercially designable. These include, without limitation, nCounter® Custom CodeSets (NanoString) (Malkov *et al.*, **2009**. *BMC Res Notes*. **2**:80; Kulkarni, **2011**. *Curr Protoc Mol Biol*. **Chapter 25**:Unit25B.10).

In one embodiment, when the expression level of more than one stroke biomarker is determined in said sample, preferably bodily fluid sample, more preferably blood sample,
25 from the subject to be diagnosed as being affected or not with a stroke, a composite score of said expression levels is calculated.

In one embodiment, the method of diagnosing a stroke according to the present invention comprises a step of comparing the signature determined from the sample, preferably the bodily fluid sample, more preferably the blood sample, of the subject, with a reference signature, as defined hereinabove.

- 5 In one embodiment, the method of diagnosing a stroke according to the present invention comprises a step of comparing the signature determined from the sample, preferably the bodily fluid sample, more preferably the blood sample, of the subject, with a predetermined reference value, as defined hereinabove.

- 10 In one embodiment, the method of diagnosing a stroke according to the present invention comprises a step of comparing the expression levels of the stroke biomarkers in the signature determined from the sample, preferably the bodily fluid sample, more preferably the blood sample, of the subject, with the expression levels of the stroke biomarkers in a reference signature, as defined hereinabove.

- 15 In one embodiment, the method of diagnosing a stroke according to the present invention comprises a step of diagnosing a stroke in the subject based on the correlation of the signature in a sample, preferably a bodily fluid sample, more preferably a blood sample, from said subject compared with the reference signature.

- 20 In one embodiment, the subject is diagnosed with a stroke when the signature determined from the sample, preferably the bodily fluid sample, more preferably the blood sample, of the subject is considered different to the reference signature, as defined hereinabove.

- In one embodiment, the signature determined from the sample, preferably the bodily fluid sample, more preferably the blood sample, of the subject is considered different to the reference signature if the expression fold change of at least 1, preferably at least 2, 3, 4, 5, 6, 7, 8 or 9 stroke biomarkers as defined herein is at least greater than about |1.1|, 25 preferably at least greater than about |1.2|, about |1.3|, about |1.4|, about |1.5|, about |1.6|, about |1.7|, about |1.8|, about |1.9|, about |2.0|, about |2.1|, about |2.2|, about |2.3|, about |2.4|, about |2.5|, about |3.0|, about |4.0|, about |5.0| or more.

In one embodiment, the signature determined from the sample, preferably the bodily fluid sample, more preferably the blood sample, of the subject is considered different to the reference signature if the expression fold change of at least 1, preferably at least 2, 3, 4, 5, 6, 7, 8 or 9 stroke biomarkers as defined herein is at least greater than about 1.1, preferably at least greater than about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9, about 2.0, about 2.1, about 2.2, about 2.3, about 2.4, about 2.5, about 3.0, about 4.0, about 5.0 or more.

In one embodiment, the signature determined from the sample, preferably the bodily fluid sample, more preferably the blood sample, of the subject is considered different to the reference signature if the $\log_2$ expression fold change of at least 1, preferably at least 2, 3, 4, 5, 6, 7, 8 or 9 stroke biomarkers as defined herein is at least greater than about |0.5|, preferably at least greater than about |0.1|, about |0.2|, about |0.3|, about |0.4|, about |0.5|, about |0.6|, about |0.7|, about |0.8|, about |0.9|, about |1.0|, about |1.1|, about |1.2|, about |1.3|, about |1.4|, about |1.5|, about |1.6|, about |1.7|, about |1.8|, about |1.9|, about |2.0|, about |2.1|, about |2.2|, about |2.3| or more.

In one embodiment, the signature determined from the sample, preferably the bodily fluid sample, more preferably the blood sample, of the subject is considered different to the reference signature if the $\log_2$ expression fold change of at least 1, preferably at least 2, 3, 4, 5, 6, 7, 8 or 9 stroke biomarkers as defined herein is at least greater than about 0.5, preferably at least greater than about 0.1, about 0.2, about 0.3, about 0.4, about 0.5, about 0.6, about 0.7, about 0.8, about 0.9, about 1.0, about 1.1, about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9, about 2.0, about 2.1, about 2.2, about 2.3 or more.

In one embodiment, the signature determined from the sample, preferably the bodily fluid sample, more preferably the blood sample, of the subject is considered different to the reference signature if the expression level of at least 1, preferably at least 2, 3, 4, 5, 6, 7, 8 or 9 stroke biomarkers as defined herein is at least about 0.5, about 1, about 1.5, about 2, about 2.5, about 3, about 3.5, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 15, about 20, about 30, about 40, about 50, or about 100 times higher than the

expression level of the same biomarker determined in the substantially healthy subject, preferably in the reference population.

In one embodiment, the subject is diagnosed with a stroke when the expression level of at least one, preferably at least 2, 3, 4, 5, 6, 7, 8, or 9 stroke biomarkers according to the invention is higher than the expression level of the same stroke biomarker in a substantially healthy subject, preferably in a reference population.

In one embodiment, the subject is diagnosed with a stroke when the composite score calculated for the expression levels of more than one, preferably at least 2, 3, 4, 5, 6, 7, 8, or 9 stroke biomarkers according to the invention, is higher than the predetermined reference value, *i.e.*, the composite score calculated for the expression levels of the same biomarkers in a substantially healthy subject, preferably in a reference population.

In one embodiment, the method of diagnosing a stroke according to the present invention comprises the steps of:

- i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from a subject to be diagnosed as being affected or not with a stroke;
- ii) determining the signature of the present invention in said sample;
- iii) comparing the signature determined in step ii) with a reference signature; and
- iv) diagnosing the subject as being affected with a stroke based on the correlation of the signature with the reference signature, as detailed hereinabove.

In one embodiment, the method of diagnosing a stroke according to the present invention comprises the steps of:

- i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from a subject to be diagnosed as being affected or not with a stroke;
- ii) determining the signature in said sample, preferably by measuring the expression level of at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or more stroke biomarkers selected from the group comprising or consisting of *HSPA1B*, *NPAS4*, *DNAJB1*, *ATF3*, *HSPB1*, *RRAD*, *NR4A1*, *CYR61*, *C-FOS*, *GADD45G*, *RGS1*, *ARC*, *EGR4*, *PTGS2*, *RGS2*, *CCL3*, *BAG3*, *EGR2*, *HSPA4L*, *ADM*, *TM4SF1*, *EGR1*, *DUSP1*, *BTG2*,

LOC715456, HMOX1, LDLR, DNAJA4, MCL1, HSPA6, GADD45B, IL6, ADFP, HES4, DUSP5, GEM and GOS2,

preferably with the proviso that the at least one or two stroke biomarkers do not consist in *DUSP1* and/or *ADM*;

- 5 iii) comparing the signature determined in step ii) with a reference signature; and
- iv) diagnosing the subject as being affected with a stroke based on the correlation of the signature with the reference signature, as detailed hereinabove.

In one embodiment, the method of diagnosing a stroke according to the present invention comprises the steps of:

- 10 i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from a subject to be diagnosed as being affected or not with a stroke;
- ii) determining the signature in said sample, preferably by measuring the expression level of at least 1, 2, 3, 4, 5, 6, 7, 8 or 9 stroke biomarkers selected from the group comprising or consisting of *PTGS2, HMOX1, LDLR, HSPA1B, GOS2, BAG3,*
- 15 *TM4SF1, DUSP1* and *ADM*,
- preferably with the proviso that the at least one or two stroke biomarkers do not consist in *DUSP1* and/or *ADM*;
- iii) comparing the signature determined in step ii) with a reference signature; and
- iv) diagnosing the subject as being affected with a stroke based on the correlation of
- 20 the signature with the reference signature, as detailed hereinabove.

In one embodiment, the method of diagnosing a stroke according to the present invention comprises the steps of:

- i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from a subject to be diagnosed as being affected or not with a stroke;
- 25 ii) determining the signature in said sample, preferably by measuring the expression level of at least three stroke biomarkers selected from the group comprising or consisting of *PTGS2, HMOX1, LDLR, HSPA1B, GOS2, BAG3, TM4SF1, DUSP1* and *ADM*;
- iii) comparing the signature determined in step ii) with a reference signature; and

- iv) diagnosing the subject as being affected with a stroke based on the correlation of the signature with the reference signature, as detailed hereinabove.

In one embodiment, the method of diagnosing a stroke according to the present invention comprises the steps of:

- 5 i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from a subject to be diagnosed as being affected or not with a stroke;
- ii) determining the signature in said sample, preferably by measuring the expression level of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *GOS2*, *BAG3* and *TM4SF1*;
- iii) comparing the signature determined in step ii) with a reference signature; and
- 10 iv) diagnosing the subject as being affected with a stroke based on the correlation of the signature with the reference signature, as detailed hereinabove.

In one embodiment, the method of diagnosing a stroke according to the present invention comprises the steps of:

- i) providing a sample, preferably a bodily fluid sample, more preferably a blood
- 15 sample, from a subject to be diagnosed as being affected or not with a stroke;
- ii) determining the signature in said sample, preferably by measuring the expression level of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *GOS2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*;
- iii) comparing the signature determined in step ii) with a reference signature; and
- 20 iv) diagnosing the subject as being affected with a stroke based on the correlation of the signature with the reference signature, as detailed hereinabove.

In one embodiment, the method of diagnosing a stroke according to the present invention comprises the steps of i) determining the expression level of at least one level of biomarker selected from the group consisting of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*,
 25 *GOS2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM* in a sample, preferably a bodily fluid sample, more preferably a blood sample, obtained from the subject ii) comparing the level determined at step i) with its predetermined reference value and iii) diagnosing a stroke when the expression level determined at step i) is higher than the predetermined reference value.

In one embodiment, the reference signature is derived or obtained from a reference population, preferably a reference comprising at least one substantially healthy subject.

In one embodiment, the method of diagnosing a stroke according to the present invention is applied to a subject who presents symptoms of stroke without having undergone the
5 routine screening to rule out all possible causes for stroke.

In one embodiment, the method of diagnosing a stroke according to the present invention can be part of the routine set of tests performed on a subject who presents symptoms of stroke, such as, without limitation, blindness in one eye, weakness in one arm or leg, weakness in one entire side of the body, dizziness, vertigo, double vision, weakness on
10 both sides of the body, difficulty speaking, slurred speech, or loss of coordination.

In one embodiment, the method of diagnosing a stroke according to the present invention can be carried out in addition of other diagnostic tools that include in particular Computed Tomography (CT) and Magnetic Resonance Imaging (MRI).

The present invention further relates to a method of determining whether a subject
15 suffering from stroke achieves a response with a therapy. The present invention further relates to a method of determining whether a subject suffering from stroke achieves a response during or after completion of a therapy.

In one embodiment, the method of determining whether a subject suffering from stroke achieves a response with a therapy comprises the steps of i) determining the expression
20 level of at least one biomarker of the present invention selected from the group consisting of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *GOS2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*, ii) comparing the expression level determined at step i) with a predetermined reference value and iii) concluding that the subject achieves a response when the level determined at step i) is lower than the predetermined reference value.

25 In one embodiment, the method of determining whether a subject suffering from stroke achieves a response with a therapy comprises steps of providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from the subject, determining the signature of the present invention in said sample and comparing said signature with a

reference signature. These steps have been detailed hereinabove in the frame of the method of diagnosing a stroke and apply *mutatis mutandis* to the present method.

In one embodiment, the method of determining whether a subject suffering from stroke achieves a response with a therapy comprises the steps of:

- 5 i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from the subject;
- ii) determining the signature of the present invention in said sample;
- iii) comparing the signature determined in step ii) with a reference signature; and
- iv) concluding that the subject achieves a response based on the correlation of the
- 10 signature with the reference signature, as detailed hereinabove.

In one embodiment, the method of determining whether a subject suffering from stroke achieves a response with a therapy comprises the steps of:

- i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from the subject;
- 15 ii) determining the signature in said sample, preferably by measuring the expression level of at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or more stroke biomarkers selected from the group comprising or consisting of *HSPA1B*, *NPAS4*, *DNAJB1*, *ATF3*, *HSPB1*, *RRAD*, *NR4A1*, *CYR61*, *C-FOS*, *GADD45G*, *RGS1*, *ARC*, *EGR4*, *PTGS2*, *RGS2*, *CCL3*, *BAG3*, *EGR2*, *HSPA4L*, *ADM*, *TM4SF1*, *EGR1*, *DUSP1*, *BTG2*,
- 20 *LOC715456*, *HMOX1*, *LDLR*, *DNAJA4*, *MCL1*, *HSPA6*, *GADD45B*, *IL6*, *ADFP*, *HES4*, *DUSP5*, *GEM* and *G0S2*;
- preferably with the proviso that the at least one or two stroke biomarkers do not consist in *DUSP1* and/or *ADM*;
- iii) comparing the signature determined in step ii) with a reference signature; and
- 25 iv) concluding that the subject achieves a response based on the correlation of the signature with the reference signature, as detailed hereinabove.

In one embodiment, the method of determining whether a subject suffering from stroke achieves a response with a therapy comprises the steps of:

- i) providing a sample, preferably a bodily fluid sample, more preferably a blood
- 30 sample, from the subject;

- ii) determining the signature in said sample, preferably by measuring the expression level of at least 1, 2, 3, 4, 5, 6, 7, 8 or 9 stroke biomarkers selected from the group comprising or consisting of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*;
- 5 preferably with the proviso that the at least one or two stroke biomarkers do not consist in *DUSP1* and/or *ADM*;
- iii) comparing the signature determined in step ii) with a reference signature; and
 - iv) concluding that the subject achieves a response based on the correlation of the signature with the reference signature, as detailed hereinabove.
- 10 In one embodiment, the method of determining whether a subject suffering from stroke achieves a response with a therapy comprises the steps of:
 - i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from the subject;
 - ii) determining the signature in said sample, preferably by measuring the expression
 - 15 level of at least three stroke biomarkers selected from the group comprising or consisting of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*;
 - iii) comparing the signature determined in step ii) with a reference signature; and
 - iv) concluding that the subject achieves a response based on the correlation of the
 - 20 signature with the reference signature, as detailed hereinabove.
- In one embodiment, the method of determining whether a subject suffering from stroke achieves a response with a therapy comprises the steps of:

 - i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from the subject;
 - 25 ii) determining the signature in said sample, preferably by measuring the expression level of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3* and *TM4SF1*;
 - iii) comparing the signature determined in step ii) with a reference signature; and
 - iv) concluding that the subject achieves a response based on the correlation of the signature with the reference signature, as detailed hereinabove.

In one embodiment, the method of determining whether a subject suffering from stroke achieves a response with a therapy comprises the steps of:

- i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from the subject;
- 5 ii) determining the signature in said sample, preferably by measuring the expression level of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*;
- iii) comparing the signature determined in step ii) with a reference signature; and
- iv) concluding that the subject achieves a response based on the correlation of the
- 10 signature with the reference signature, as detailed hereinabove.

In one embodiment, the reference signature is derived or obtained from a previous measurement of expression levels of stroke biomarkers according to the invention, in a reference sample derived from the same subject, such as, for example, the expression levels of stroke biomarkers measured before the start of a therapy.

- 15 In one embodiment, it is concluded that the subject achieves a response when the signature determined from the sample, preferably the bodily fluid sample, more preferably the blood sample, of the subject is considered different to the reference signature, as defined hereinabove.

- In one embodiment, the signature determined from the sample, preferably the bodily fluid
- 20 sample, more preferably the blood sample, of the subject is considered different to the reference signature if the expression fold change of at least 1, preferably at least 2, 3, 4, 5, 6, 7, 8 or 9 stroke biomarkers as defined herein is at least lower than about |1.1|, preferably at least lower than about |1.2|, about |1.3|, about |1.4|, about |1.5|, about |1.6|, about |1.7|, about |1.8|, about |1.9|, about |2.0|, about |2.1|, about |2.2|, about |2.3|, about
 - 25 |2.4|, about |2.5|, about |3.0|, about |4.0|, about |5.0| or more.

In one embodiment, the signature determined from the sample, preferably the bodily fluid sample, more preferably the blood sample, of the subject is considered different to the reference signature if the expression fold change of at least 1, preferably at least 2, 3, 4, 5, 6, 7, 8 or 9 stroke biomarkers as defined herein is at least lower than about 1.1,

preferably at least lower than about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9, about 2.0, about 2.1, about 2.2, about 2.3, about 2.4, about 2.5, about 3.0, about 4.0, about 5.0 or more.

In one embodiment, the signature determined from the sample, preferably the bodily fluid sample, more preferably the blood sample, of the subject is considered different to the reference signature if the $\log_2$ expression fold change of at least 1, preferably at least 2, 3, 4, 5, 6, 7, 8 or 9 stroke biomarkers as defined herein is at least lower than about |0.1|, preferably at least lower than about |0.2|, about |0.3|, about |0.4|, about |0.5|, about |0.6|, about |0.7|, about |0.8|, about |0.9|, about |1.0|, about |1.1|, about |1.2|, about |1.3|, about |1.4|, about |1.5|, about |1.6|, about |1.7|, about |1.8|, about |1.9|, about |2.0|, about |2.1|, about |2.2|, about |2.3| or more.

In one embodiment, the signature determined from the sample, preferably the bodily fluid sample, more preferably the blood sample, of the subject is considered different to the reference signature if the $\log_2$ expression fold change of at least 1, preferably at least 2, 3, 4, 5, 6, 7, 8 or 9 stroke biomarkers as defined herein is at least lower than about 0.1, preferably at least lower than about 0.2, about 0.3, about 0.4, about 0.5, about 0.6, about 0.7, about 0.8, about 0.9, about 1.0, about 1.1, about 1.2, about 1.3, about 1.4, about 1.5, about 1.6, about 1.7, about 1.8, about 1.9, about 2.0, about 2.1, about 2.2, about 2.3 or more.

In one embodiment, the signature determined from the sample, preferably the bodily fluid sample, more preferably the blood sample, of the subject is considered different to the reference signature if the expression level of at least 1, preferably at least 2, 3, 4, 5, 6, 7, 8 or 9 stroke biomarkers as defined herein is at least about 0.5, about 1, about 1.5, about 2, about 2.5, about 3, about 3.5, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 15, about 20, about 30, about 40, about 50, or about 100 times lower than the expression level of the same biomarker determined in the substantially healthy subject, preferably in the reference population.

In one embodiment, the signature determined from the sample, preferably the bodily fluid sample, more preferably the blood sample, of the subject is considered different to the

reference signature if the expression level of at least one, preferably at least 2, 3, 4, 5, 6, 7, 8, or 9 stroke biomarkers according to the invention is not higher than the expression level of the same stroke biomarker in a substantially healthy subject, preferably in a reference population.

- 5 In one embodiment, the signature determined from the sample, preferably the bodily fluid sample, more preferably the blood sample, of the subject is considered different to the reference signature if the composite score calculated for the expression levels of more than one, preferably at least 2, 3, 4, 5, 6, 7, 8, or 9 stroke biomarkers according to the invention, is not higher than the predetermined reference value, *i.e.*, the composite score
- 10 calculated for the expression levels of the same biomarkers in a substantially healthy subject, preferably in a reference population.

The method of determining whether a subject suffering from stroke achieves a response with a therapy is thus particularly suitable for discriminating “responders” from “non-responders”.

- 15 As used herein, the term “**responder**” refers to a subject or patient that will achieve a therapeutic response, *i.e.*, a subject in whom stroke is reduced, alleviated or cured. According to the invention, the responders have an objective response and therefore the term does not encompass subjects or patients having a stabilized stroke such that the disease is not progressing after the therapy.
- 20 As used herein, the term “**non-responder**” (or refractory) includes subjects or patients for whom stroke does not show reduction or improvement after the therapy. According to the invention, the term “non-responder” also includes subjects or patients having a stabilized stroke.

- Typically, the characterization of the subject as being a responder or non-responder can
- 25 be performed by reference to a standard or a training set. The standard may be the profile of a subject who is known to be a responder or non-responder, or alternatively may be a numerical value. Such predetermined standards may be provided in any suitable form, such as a printed list or diagram, computer software program, or other media. In some embodiments, the predetermined value is the expression level determined before the

therapy. When it is concluded that the patient is a non-responder, the physician could take the decision to stop the therapy to avoid any further adverse sides effects.

In one embodiment, for monitoring purposes, expression levels can be measured at multiple time points, for example, twice, 3 times, 4 times, 5 times, 6 times, 7 times, 8
5 times, 9 times, 10 times or more. In this manner, monitoring of expression levels over time following or during therapy can provide a measure of the success of treatment, as it is to be expected that expression levels will decrease faster in subjects with successful treatment relative to an untreated subject for which expressions levels may decrease, albeit more slowly.

10 Typically, the therapy consists in any method well known by the skilled artisan. Treatment options include, but are not limited to, endovascular procedures and surgery, such as thrombectomy.

As used, herein the term “**thrombectomy**” defines any surgical and/or mechanical removal or breakdown of a clot. Typically, three classes of mechanical thrombectomy
15 devices are known: coil retrievers, aspiration devices, and stent retrievers. Other devices and methods, currently under development, are also included in the definition of thrombectomy used herein. Typically, a catheter is sent to the blood flow blockage site to remove the blood clot. Combination of thrombectomy and thrombolysis can be administered to the patient. Additionally, ischemic stroke can also be treated by
20 administering a thrombolytic agent to dissolve the blood clot (“thrombolysis”).

Antithrombotic agents are further divided into the following three subtypes: anticoagulants, antiplatelet drugs, and thrombolytic drugs.

Examples of anticoagulants include, but are not limited to, coumarins, heparin, warfarin, acenocoumarol, phenprocoumon, atromentin, phenindione, fondaparinux, idraparinix,
25 direct factor Xa inhibitors, direct thrombin inhibitors, antithrombin protein therapeutics, batroxobin, and hementin.

Examples of antiplatelet drugs include, but are not limited to, irreversible cyclooxygenase inhibitors (*e.g.*, aspirin or triflusal), adenosine diphosphate receptor inhibitors (*e.g.*,

clopidogrel, prasugrel, ticagrelor, or ticlopidine), phosphodiesterase inhibitors (*e.g.*, cilostazol), glycoprotein IIB/IIIA inhibitors (*e.g.*, abciximab, eptifibatide, or tirofiban), adenosine reuptake inhibitors (*e.g.*, dipyridamole), and thromboxane inhibitors (*e.g.*, thromboxane synthase inhibitors or thromboxane receptor antagonists).

- 5 Examples of thrombolytic drugs include, but are not limited to, tissue plasminogen activator t-PA (alteplase and others (desmoteplase, reteplase, tenecteplase, ...), anistreplase, streptokinase, and urokinase. t-PA can be optionally administered during the endovascular procedures.

In one embodiment, the therapy consists in administering a neuroprotective agent.

- 10 Examples of neuroprotective agents typically include, without limitation, anti-free radicals, anti-glutamate agents, and CDK inhibitors.

- In some embodiments, the neuroprotective agent is a cyclin-dependent kinase (CDK) inhibitor. Known CDK inhibitors may be classified according to their ability to inhibit CDKs in general or according to their selectivity for a specific CDK. Flavopiridol, for example, acts as a “pan” CDK antagonist and is not particularly selective for a specific CDK (Dai & Grant, **2003**. *Curr Opin Pharmacol.* **3(4)**:362-70). Purine-based CDK inhibitors, such as olomoucine, roscovitine, purvanolols and CGP74514A, are known to exhibit a greater selectivity for CDKs 1, 2 and 5, but show no inhibitory activity against CDKs 4 and 6 (Dai & Grant, **2003**. *Curr Opin Pharmacol.* **3(4)**:362-70). Furthermore, it
- 15 has been demonstrated that purine-based CDK inhibitors such as S-roscovitine can exert anti-apoptotic effects in the nervous system (O’Hare *et al.*, **2002**. *Pharmacol Ther.* **93(2-3)**:135-43; Timsit & Menn, **2012**. *Clin Pharmacol Ther.* **91(2)**:327-32; Gutiérrez-Vargas *et al.*, **2017**. *J Cereb Blood Flow Metab.* **37(6)**:2208-2223) or prevent neuronal death in neurodegenerative diseases, such as Alzheimer’s disease (Filgueira de Azevedo *et al.*,
20 **2002**. *Biochem Biophys Res Commun.* **297(5)**:1154-8; Knockaert *et al.*, **2002**. *Trends Pharmacol Sci.* **23(9)**:417-25).

In some embodiments, the therapy consists in hypothermia (*e.g.*, Kurisu & Yenari, **2018**. *Neuropharmacology.* **134(Pt B)**:302-309).

The present invention further relates to a method of determining whether a subject is at risk of having stroke comprising the steps of i) determining the expression level of at least one level of biomarker selected from the group consisting of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *GOS2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM* in a sample, preferably a bodily fluid sample, more preferably a blood sample, obtained from the subject ii) comparing the level determined at step i) with its predetermined reference value and iii) concluding that the subject is at risk of having stroke when the expression level determined at step is higher than the predetermined reference value.

In one embodiment, the method of determining whether a subject is at risk of having stroke comprises steps of providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from the subject, determining the signature of the present invention in said sample and comparing said signature with a reference signature. These steps have been detailed hereinabove in the frame of the method of diagnosing a stroke and apply *mutatis mutandis* to the present method.

In one embodiment, the method of determining whether a subject is at risk of having stroke comprises the steps of:

- i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from the subject;
- ii) determining the signature of the present invention in said sample;
- iii) comparing the signature determined in step ii) with a reference signature; and
- iv) concluding that the subject is at risk of having stroke based on the correlation of the signature with the reference signature, as detailed hereinabove.

In one embodiment, the method of determining whether a subject is at risk of having stroke comprises the steps of:

- i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from the subject;
- ii) determining the signature in said sample, preferably by measuring the expression level of at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or more stroke biomarkers selected from the group comprising or consisting of *HSPA1B*, *NPAS4*, *DNAJB1*, *ATF3*, *HSPB1*, *RRAD*, *NR4A1*, *CYR61*, *C-FOS*, *GADD45G*, *RGS1*, *ARC*, *EGR4*, *PTGS2*, *RGS2*,

CCL3, BAG3, EGR2, HSPA4L, ADM, TM4SF1, EGR1, DUSP1, BTG2, LOC715456, HMOX1, LDLR, DNAJA4, MCL1, HSPA6, GADD45B, IL6, ADFP, HES4, DUSP5, GEM and GOS2;

preferably with the proviso that the at least one or two stroke biomarkers do not consist in *DUSP1* and/or *ADM*;

- iii) comparing the signature determined in step ii) with a reference signature; and
- iv) concluding that the subject is at risk of having stroke based on the correlation of the signature with the reference signature, as detailed hereinabove.

In one embodiment, the method of determining whether a subject is at risk of having stroke comprises the steps of:

- i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from the subject;
- ii) determining the signature in said sample, preferably by measuring the expression level of at least 1, 2, 3, 4, 5, 6, 7, 8 or 9 stroke biomarkers selected from the group comprising or consisting of *PTGS2, HMOX1, LDLR, HSPA1B, GOS2, BAG3, TM4SF1, DUSP1* and *ADM*;
- preferably with the proviso that the at least one or two stroke biomarkers do not consist in *DUSP1* and/or *ADM*;
- iii) comparing the signature determined in step ii) with a reference signature; and
- iv) concluding that the subject is at risk of having stroke based on the correlation of the signature with the reference signature, as detailed hereinabove.

In one embodiment, the method of determining whether a subject is at risk of having stroke comprises the steps of:

- i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from the subject;
- ii) determining the signature in said sample, preferably by measuring the expression level of at least three stroke biomarkers selected from the group comprising or consisting of *PTGS2, HMOX1, LDLR, HSPA1B, GOS2, BAG3, TM4SF1, DUSP1* and *ADM*;
- iii) comparing the signature determined in step ii) with a reference signature; and

- iv) concluding that the subject is at risk of having stroke based on the correlation of the signature with the reference signature, as detailed hereinabove.

In one embodiment, the method of determining whether a subject is at risk of having stroke comprises the steps of:

- 5 i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from the subject;
- ii) determining the signature in said sample, preferably by measuring the expression level of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *GOS2*, *BAG3* and *TM4SF1*;
- iii) comparing the signature determined in step ii) with a reference signature; and
- 10 iv) concluding that the subject is at risk of having stroke based on the correlation of the signature with the reference signature, as detailed hereinabove.

In one embodiment, the method of determining whether a subject is at risk of having stroke comprises the steps of:

- i) providing a sample, preferably a bodily fluid sample, more preferably a blood
- 15 sample, from the subject;
- ii) determining the signature in said sample, preferably by measuring the expression level of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *GOS2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*;
- iii) comparing the signature determined in step ii) with a reference signature; and
- 20 iv) concluding that the subject is at risk of having stroke based on the correlation of the signature with the reference signature, as detailed hereinabove.

In one embodiment, the reference signature is derived or obtained from a reference population, preferably a reference comprising at least one substantially healthy subject.

- 25 In one embodiment, the subject suffers from or has suffered from or has been diagnosed with a cardiovascular disease (*e.g.*, atherosclerosis, hypertension...) that can lead to stroke.

In one embodiment, the subject has experienced a stroke and the method of the present invention of the present invention is particularly suitable for predicting a recurrent stroke.

The method of determining whether a subject is at risk of having stroke is thus particularly suitable for providing a prognosis and thus identifying subjects at risk and then take all therapeutic interventions for preventing stroke.

As used herein, the term “**risk**” relates to the probability that an event will occur over a specific time period and can mean a subject’s “absolute risk” or “relative risk”.

“**Absolute risk**” can be measured with reference to either actual observation post-measurement for the relevant time cohort, or with reference to index values developed from statistically valid historical cohorts that have been followed for the relevant time period.

10 “**Relative risk**” refers to the ratio of absolute risks of a subject compared either to the absolute risks of low risk cohorts or an average population risk, which can vary by how clinical risk factors are assessed. Odds ratios, the proportion of positive events to negative events for a given test result, are also commonly used (odds are according to the formula $p / (1 - p)$ where p is the probability of event and $(1 - p)$ is the probability of no event) to
15 no-conversion.

“**Risk evaluation**” or “**evaluation of risk**” in the context of the present invention encompasses making a prediction of the probability, odds, or likelihood that an event or disease state may occur, the rate of occurrence of the event or conversion from one disease state to another. Risk evaluation can also comprise prediction of future clinical
20 parameters, traditional laboratory risk factor values, or other indices of relapse, either in absolute or relative terms in reference to a previously measured population.

The determining whether a subject is at risk of having stroke may be used to make continuous or categorical measurements of the risk of conversion, thus diagnosing and defining the risk spectrum of a category of subjects defined as being at risk of conversion.
25 In the categorical scenario, the invention can be used to discriminate between normal and other subject cohorts at higher risk. In one embodiment, the present invention may be used so as to discriminate those at risk from normal.

- The present invention further relates to a method of determining the prognosis of a subject suffering from stroke comprising i) determining the expression level of at least one level of biomarker selected from the group consisting of *DUSP1*, *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *GOS2*, *BAG3*, *ADM*, and *TM4SF1* in a sample, preferably a bodily fluid sample, more preferably a blood sample, obtained from the subject ii) comparing the level determined at step i) with its predetermined reference value and iii) concluding that the patient has a good prognosis when the level determined at step i) is lower than the predetermined reference value or concluding that the patient has a poor prognosis when the level determined at step i) is higher than the predetermined reference value.
- 10 In one embodiment, the method of determining the prognosis of a subject suffering from stroke comprises steps of providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from the subject, determining the signature of the present invention in said sample and comparing said signature with a reference signature. These steps have been detailed hereinabove in the frame of the method of diagnosing a stroke
- 15 and apply *mutatis mutandis* to the present method.

In one embodiment, the method of determining the prognosis of a subject suffering from stroke comprises the steps of:

- i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from the subject;
- 20 ii) determining the signature of the present invention in said sample;
- iii) comparing the signature determined in step ii) with a reference signature; and
- iv) concluding that the subject the patient has a good prognosis based on the correlation of the signature with the reference signature, as detailed hereinabove.

In one embodiment, the method of determining the prognosis of a subject suffering from stroke comprises the steps of:

- 25 i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from the subject;
- ii) determining the signature in said sample, preferably by measuring the expression level of at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or more stroke biomarkers selected from the
- 30 group comprising or consisting of *HSPA1B*, *NPAS4*, *DNAJB1*, *ATF3*, *HSPB1*,

RRAD, NR4A1, CYR61, C-FOS, GADD45G, RGS1, ARC, EGR4, PTGS2, RGS2, CCL3, BAG3, EGR2, HSPA4L, ADM, TM4SF1, EGR1, DUSP1, BTG2, LOC715456, HMOX1, LDLR, DNAJA4, MCL1, HSPA6, GADD45B, IL6, ADFP, HES4, DUSP5, GEM and GOS2;

- 5 preferably with the proviso that the at least one or two stroke biomarkers do not consist in *DUSP1* and/or *ADM*;
- iii) comparing the signature determined in step ii) with a reference signature; and
- iv) concluding that the subject the patient has a good prognosis based on the correlation of the signature with the reference signature, as detailed hereinabove.
- 10 In one embodiment, the method of determining the prognosis of a subject suffering from stroke comprises the steps of:
- i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from the subject;
- ii) determining the signature in said sample, preferably by measuring the expression
- 15 level of at least 1, 2, 3, 4, 5, 6, 7, 8 or 9 stroke biomarkers selected from the group comprising or consisting of *PTGS2, HMOX1, LDLR, HSPA1B, GOS2, BAG3, TM4SF1, DUSP1* and *ADM*;
- preferably with the proviso that the at least one or two stroke biomarkers do not consist in *DUSP1* and/or *ADM*;
- 20 iii) comparing the signature determined in step ii) with a reference signature; and
- iv) concluding that the subject the patient has a good prognosis based on the correlation of the signature with the reference signature, as detailed hereinabove.

In one embodiment, the method of determining the prognosis of a subject suffering from stroke comprises the steps of:

- 25 i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from the subject;
- ii) determining the signature in said sample, preferably by measuring the expression level of at least three stroke biomarkers selected from the group comprising or consisting of *PTGS2, HMOX1, LDLR, HSPA1B, GOS2, BAG3, TM4SF1, DUSP1*
- 30 and *ADM*;

- iii) comparing the signature determined in step ii) with a reference signature; and
- iv) concluding that the subject the patient has a good prognosis based on the correlation of the signature with the reference signature, as detailed hereinabove.

In one embodiment, the method of determining the prognosis of a subject suffering from stroke comprises the steps of:

- i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from the subject;
- ii) determining the signature in said sample, preferably by measuring the expression level of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *GOS2*, *BAG3* and *TM4SF1*;
- 10 iii) comparing the signature determined in step ii) with a reference signature; and
- iv) concluding that the subject the patient has a good prognosis based on the correlation of the signature with the reference signature, as detailed hereinabove.

In one embodiment, the method of determining the prognosis of a subject suffering from stroke comprises the steps of:

- 15 i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from the subject;
- ii) determining the signature in said sample, preferably by measuring the expression level of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *GOS2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*;
- 20 iii) comparing the signature determined in step ii) with a reference signature; and
- iv) concluding that the subject the patient has a good prognosis based on the correlation of the signature with the reference signature, as detailed hereinabove.

In one embodiment, the reference signature is derived or obtained from a reference population, preferably a reference comprising at least one substantially healthy subject.

- 25 In one embodiment, the reference signature is derived or obtained from a previous measurement of expression levels of stroke biomarkers according to the invention, in a reference sample derived from the same subject, such as, for example, the expression levels of stroke biomarkers measured after a stroke, preferably no less than 6 hours, 12

hours, 18 hours, 24 hours, 36 hours, 48 hours, 60 hours, 72 hours, 84 hours or 96 hours after a stroke.

As used herein, the term “**prognosis**” refers to a prediction of the probable outcome of stroke, *i.e.*, the course of stroke or survival time the patient.

- 5 The term “**good prognosis**”, as used herein, refers to greater than average likelihood of survival for a patient suffering from stroke as compared to other members of the same gender suffering from the same condition.

10 The term “**poor prognosis**” as used herein, refers to a less than average likelihood of survival for a patient suffering from stroke as compared to other members of the same gender suffering from the same condition.

The present invention further relates to a method of treating a subject affected with a stroke.

15 In one embodiment, the method of treating a subject affected with a stroke comprises steps of providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from the subject, determining the signature of the present invention in said sample and comparing said signature with a reference signature. These steps have been detailed hereinabove in the frame of the method of diagnosing a stroke and apply *mutatis mutandis* to the present method.

20 In one embodiment, the method of treating a subject affected with a stroke comprises the steps of:

- i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from said subject;
- ii) determining the signature of the present invention in said sample;
- iii) comparing the signature determined in step ii) with a reference signature;
- 25 iv) diagnosing the subject as being affected with a stroke based on the correlation of the signature with the reference signature, as detailed hereinabove; and
- v) treating the subject if said subject was diagnosed as being affected with a stroke in step iv).

In one embodiment, the method of treating a subject affected with a stroke comprises the steps of:

- i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from said subject;
- 5 ii) determining the signature in said sample, preferably by measuring the expression level of at least 1, 2, 3, 4, 5, 6, 7, 8, 9 or more stroke biomarkers selected from the group comprising or consisting of *HSPA1B*, *NPAS4*, *DNAJB1*, *ATF3*, *HSPB1*, *RRAD*, *NR4A1*, *CYR61*, *C-FOS*, *GADD45G*, *RGS1*, *ARC*, *EGR4*, *PTGS2*, *RGS2*, *CCL3*, *BAG3*, *EGR2*, *HSPA4L*, *ADM*, *TM4SF1*, *EGR1*, *DUSP1*, *BTG2*,
10 *LOC715456*, *HMOX1*, *LDLR*, *DNAJA4*, *MCL1*, *HSPA6*, *GADD45B*, *IL6*, *ADFP*, *HES4*, *DUSP5*, *GEM* and *G0S2*;
preferably with the proviso that the at least one or two stroke biomarkers do not consist in *DUSP1* and/or *ADM*;
- iii) comparing the signature determined in step ii) with a reference signature;
- 15 iv) diagnosing the subject as being affected with a stroke based on the correlation of the signature with the reference signature, as detailed hereinabove; and
- v) treating the subject if said subject was diagnosed as being affected with a stroke in step iv).

In one embodiment, the method of treating a subject affected with a stroke comprises the steps of:

- i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from said subject;
- ii) determining the signature in said sample, preferably by measuring the expression level of at least 1, 2, 3, 4, 5, 6, 7, 8 or 9 stroke biomarkers selected from the group
25 comprising or consisting of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*;
preferably with the proviso that the at least one or two stroke biomarkers do not consist in *DUSP1* and/or *ADM*;
- iii) comparing the signature determined in step ii) with a reference signature;
- 30 iv) diagnosing the subject as being affected with a stroke based on the correlation of the signature with the reference signature, as detailed hereinabove; and

- v) treating the subject if said subject was diagnosed as being affected with a stroke in step iv).

In one embodiment, the method of treating a subject affected with a stroke comprises the steps of:

- 5 i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from said subject;
- ii) determining the signature in said sample, preferably by measuring the expression level of at least three stroke biomarkers selected from the group comprising or consisting of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1*
10 and *ADM*;
- iii) comparing the signature determined in step ii) with a reference signature;
- iv) diagnosing the subject as being affected with a stroke based on the correlation of the signature with the reference signature, as detailed hereinabove; and
- 15 v) treating the subject if said subject was diagnosed as being affected with a stroke in step iv).

In one embodiment, the method of treating a subject affected with a stroke comprises the steps of:

- i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from said subject;
- 20 ii) determining the signature in said sample, preferably by measuring the expression level of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3* and *TM4SF1*;
- iii) comparing the signature determined in step ii) with a reference signature;
- iv) diagnosing the subject as being affected with a stroke based on the correlation of the signature with the reference signature, as detailed hereinabove; and
- 25 v) treating the subject if said subject was diagnosed as being affected with a stroke in step iv).

In one embodiment, the method of treating a subject affected with a stroke comprises the steps of:

- 30 i) providing a sample, preferably a bodily fluid sample, more preferably a blood sample, from said subject;

- ii) determining the signature in said sample, preferably by measuring the expression level of *PTGS2*, *HMOX1*, *LDLR*, *HSPA1B*, *G0S2*, *BAG3*, *TM4SF1*, *DUSP1* and *ADM*;
- iii) comparing the signature determined in step ii) with a reference signature;
- 5 iv) diagnosing the subject as being affected with a stroke based on the correlation of the signature with the reference signature, as detailed hereinabove; and
- v) treating the subject if said subject was diagnosed as being affected with a stroke in step iv).

In one embodiment, the reference signature is derived or obtained from a reference
10 population, preferably a reference comprising at least one substantially healthy subject.

Examples of suitable treatments and therapies for subjects diagnosed as being affected with a stroke are well known in the art, and have been detailed hereinabove.

The invention will be further illustrated by the following example and figures. However,
this example should not be interpreted in any way as limiting the scope of the present
15 invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a set of 3 graphs showing the correlation between mRNA levels and microarray intensity values for (a) *HSPA1B*, (b) *GADD45G* and (c) *CDKN1A* by qRT-
20 PCR. The *x*-axis shows the log₂ ratio of qRT-PCR mRNA normalized for *SMC2* and the *y*-axis shows the oligonucleotide microarray signal. *P*-value from Mann-Whitney U test.

Figure 2 is a set of two Venn diagrams of the most significant overlap between the two sets of genes differentially expressed in the brain and in the blood. Upper panel shows down-regulated genes; lower panel shows up-regulated genes.

25 **Figure 3** illustrates the blood gene intensity kinetic in the blood for the 9 top differentially expressed genes in the male rhesus macaque S1 between different time points: before (*T*₀) and after (*T*₁, *T*₂, *T*₃) ischemia.

Figure 4 illustrates the blood gene intensity kinetic in the blood for the 9 top differentially expressed genes in the male rhesus macaque S2 between different time points: before (T_0) and after (T_1 , T_2 , T_3) ischemia.

5 **EXAMPLES**

The present invention is further illustrated by the following examples.

Example 1: identification of a common signature between the ischemic brain and the blood in a thrombotic Rhesus macaque model

Methods

10 ***Animal experiment***

Experiments were performed in 2 male rhesus macaques (*Macaca mulatta*) aged 12-13 years old, and with body weights ranging from 16.5 to 17.2 kg. In the following, these two monkeys will be noted S1 and S2.

15 An experimental protocol was submitted to the Regional Ethics Committee for Animal Experimentation (Normandy) and approval was granted to conduct the study (referral No. N/02-03-08/03/02-11). Experiments were performed by licensed investigators (C.O.) and in accordance with French and European ethical laws and guidelines for the care and use of laboratory animals (Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes).

20 During the course of the present studies, the monkeys were housed at the Cyceron Research Centre (Establishment for Animal Experimentation, agreement No. B14118001) in individual cages maintained at 24 °C with 50% relative humidity on a 12-hour/12-hour light/dark cycle and were fed with commercial chow supplemented with fresh fruits and water *ad libitum*. Throughout the duration of these studies, a veterinary

25 surgeon was available to oversee the well-being of the animals.

Animal model

Thrombotic Rhesus Macaque model

Experiments were performed as previously described (Gauberti *et al.*, **2012**. *Cerebrovasc Dis.* **33(4)**:329-39).

- 5 Briefly, after general anaesthesia, following enucleation, the right middle cerebral artery (MCA) was exposed. Two sutures were positioned to isolate M1 branch into which the micropipette was inserted and human thrombin injected. About 600 μL of thrombin were injected at a concentration of 1 U/ μL . A first injection of 100 μL was applied followed by 6 injection of 50 μL at 2 minutes of interval, and then the proximal suture was removed
10 and the remaining thrombin was injected. The distal suture was then removed after 15 minutes.

The total procedure for thrombin injection took about 30 minutes.

Anaesthesia and control of physiologic parameters

- Animals were tranquilized with ketamine (0.1 mg/kg; IM; Imalgene®). Gaseous
15 anaesthesia was induced by sevoflurane (2.5%; Sevorane®) in 100% oxygen. Muscular relaxation was obtained by atracurium (0.5 mg/kg; IV; Tracrium®) through a saphenous vein.

- Monkeys were mechanically ventilated with positive intermittent pressure at a fixed respiratory frequency of 22 per minute. The tidal volume (V_T) was adjusted to obtain a
20 normocapnia ($\text{PaCO}_2 = 38\text{-}42$ mmHg). Anaesthesia was maintained by sevoflurane with 66% of nitrous oxide.

- During surgery, sevoflurane concentration was increased to 3% then decreased to 1.5-2% during magnetic resonance imaging (MRI) procedure. Intravenous atracurium perfusion was set at 0.75 mg/kg/h. The animals were positioned in a stereotactic frame before
25 surgery.

In vivo MRI acquisition

Monkeys were studied in a 3T clinical MRI (Philips Sense Flex M).

Imaging was performed in the axial and coronal plane and included the following sequences: 3D-time-of-flight angiography, T2-weighted, fluid attenuation inversion recovery (FLAIR), diffusion-weighted imaging (DWI), and pre and post-contrast T1-weighted and perfusion-weighted imaging (PWI).

Blood samples

Blood samples were obtained at T_0 before surgery but after general anaesthesia, and at $T_1 = +2h$, $T_2 = +3.5h$ and $T_3 = +4.5h$ after MCA occlusion.

10 For each blood sample, 2.5 mL were drawn for RNA. Blood samples were collected using PAXgenes Blood RNA tubes (PreAnalytix).

Brain samples

12 brain samples were taken from each monkey. In each monkey, 6 samples were taken near the infarction site, and 6 samples were taken from the corresponding location in the other hemisphere. A total of 12 ischemic and 12 non-ischemic brain samples were thus analysed.

The two monkeys were operated and sacrificed about 5.5 hours after onset of occlusion (S1: 5h12; S2: 5h35). An MRI was performed between 3 and 4 hours after occlusion onset (S1: 3h10; S2: 3h05). Animals were infused by intra-cardiac injection after thoracotomy by about a total volume of 8 L of 4°C saline serum. The brain was then removed after craniotomy and placed into a mold specifically designed for coronal brain slicing. Each cut of brain was further placed onto a grid to be able to identify x - and y -coordinates on the ischemic and contralateral hemisphere. Arbitrarily on the grid, Roman numerals were for the right hemispheric ischemic side, and Arabic numerals for the left non-ischemic hemisphere side.

Ischemia was visible to the naked eye (**data not shown**) and confirmed by hypometabolic ischemic tissue treated by tetrazolium which appeared white as compared to the pinkish staining obtained in the non-ischemic contralateral hemisphere.

Three ischemic cortical samples per animal were prepared: one potentially corresponding to the core, and two from the edge, corresponding potentially to a penumbral region. Three homologous samples from the contralateral hemisphere per animal were also prepared.

TTC staining

To evaluate metabolic activity and therefore ischemic tissue, brain samples adjacent tissue to be studied for transcriptomics were stained with 1% tetrazolium chloride (TTC).

Total RNA extraction

Total RNA was isolated from cerebral cortex using the RNeasy Microarray Tissue kit following the manufacturer's instructions (Qiagen). RNA integrity was assessed with the Agilent 2100 BioAnalyzer (Agilent Technologies) by measuring the RIN (RNA integrity number) (Schroeder *et al.*, **2006**. *BMC Mol Biol.* **7**:3). Measured RIN was between 6.3 and 8.8.

Total RNA was isolated from blood samples using the PAXgene Blood RNA Kit (PreAnalytix). Globin mRNA was removed from total RNA using the GlobinClear kit (Ambion). Measured RIN was between 7.8 and 10.

Macaca expression microarray and choice of samples

RNA from cerebral cortex (30 ng) and from blood sample (30 ng) were labelled using the Low Input Quick Amp WT Labeling kit (Agilent Technologies). RNA spike-in controls were used to adjust possible dye effects. RNA was converted to cDNA using reverse transcriptase and WT primers (T7 promoter primer and random primer with a T7 promoter). T7 RNA polymerase was used for the synthesis and labelling of cRNA with Cy3. The fluorescent-labelled cRNA probes were purified using the RNeasy mini kit (Qiagen).

An equal amount (3.75 µg) of Cy3 cRNA probes were hybridized on 4x44K Agilent DNA chip (catalogue number: G2519F, *Macacca mulatta*). Hybridization was performed for 17 hours, rotating at 10 rpm at 65 °C. Then, samples were washed and dried according to the manufacturer's instructions.

- 5 Hybridization images were obtained using Agilent DNA microarray scanner and intensity data was extracted using Feature Extraction software (Agilent Technologies). This array contains 43803 rhesus macaque monkey probes. These probes are sourced from RefSeq (Release 37, Oct 2009), Unigene (Release 13, Oct 2009), UCSC MRNA (Oct 2009), Ensembl (Release 56, Sep 2009), UCSC RheMac2 (Jan 2006). Many probes are predicted
10 based on orthologous human genes. Additionally, some probes are annotated only as *Macaca mulatta* cDNA, and other than what can be inferred from homology with human the functions are not known. Analysis was performed in collaboration with Genosplice (Evry, France), a company specialized in transcriptomic analysis.

Microarray analysis

- 15 Differential expression analysis of the Agilent microarray expression data was performed using limma from the Bioconductor project (Smyth, **2004**. *Stat Appl Genet Mol Biol.* **3**:Article3). Raw data were normalized, first by performing background correction and then by normalizing between arrays for all brain and blood samples also using limma. Probes were annotated using Agilent's array information provided on Array Express
20 (<http://www.ebi.ac.uk/arrayexpress/arrays/A-GEOD-9861/?page=71&pagesize=100&sortby=organism&sortorder=descending>) and gene information provided by Genosplice.

- The limma package performs differential expression analysis by first fitting the expression data of each gene to a linear model. It then utilizes Empirical Bayes (eBayes)
25 to borrow information across genes, which allows us to perform analysis across a small number of arrays.

All differential expression analyses of the brain microarray data were done using limma's eBayes method (Smyth, **2005**. limma: Linear Models for Microarray Data. In: Gentleman

et al. (Eds), *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*. Statistics for Biology and Health. New York, NY: Springer).

Statistics

Comparison of highly expressed gene in the brain

- 5 The two monkeys were analysed separately. Genes were labelled as “highly differentially expressed” if they had a fold change of more than 2 (*i.e.*, a $\log_2$ fold change greater than 1) in both monkeys when comparing ischemic and non-ischemic brain samples, and had a fold change of less than 2 when comparing ischemic samples between monkeys or when comparing non-ischemic samples between monkeys.

10 *Comparison of highly expressed gene in the blood*

- Blood samples were analysed by comparing the pre-occlusion sample to each post-occlusion sample. The two monkeys were analysed separately. The time point with the largest fold change was selected for further analysis and called “S1 max blood” and “S2 max blood” (**Table 1**). Genes were labelled as “highly differentially expressed” if they
- 15 had a fold change more than 1.5 in both monkeys when comparing the pre-occlusion sample to “S1 max blood” and “S2 max blood”.

Only highly differentially expressed genes in both ischemic brain and post-occlusion blood samples as defined above were compared and used to identify common highly differentially expressed genes.

20 ***Quality control of data***

Internal validation

- A probe-specific two step TaqMan R Gene Expression Assay was used to validate microarray results (Applied Biosystems). Genes for validation were chosen based on ranking of differential expression and biological annotation relevant to ischemia. We
- 25 chose *SMC2* for normalization across target genes, for this gene showed a constant expression in ischemic and non-ischemic tissues samples. Gene Expression Assay probe

IDs were as follows: *HSPA1B* (A 01 P010726), *GADD45G* (A 01 P018040), *CDKN1A* (A 01 P002585), *SMC2* (A 01 P019124).

100 ng of total RNA of each sample was used to generate cDNA using the SuperScript® III First-Strand Synthesis Kit (ThermoFisher scientific) following the manufacturer's protocol. Real-time PCR reactions were carried out on the Roche LightCycler R480 System. Gene expressions were compared between ischemic and non-ischemic tissues using the comparative C_T method ($\Delta\Delta C_T$ Method) with the Mann-Whitney U test (Wilcoxon), utilizing Prism software v6.0c (GraphPad, La Jolla, CA).

External validation

10 We attempted to verify our results using data from a study by Cook *et al.* (2012. *Nature*. **483**(7388):213-7). This study also examined stroke transcriptomics in gyrencephalic primate, however using cynomolgus macaques, a close relative of *Macaca mulatta* (Street *et al.*, 2007. *BMC Genomics*. **8**:480). In order to validate the results found in *Macaca mulatta*, we analysed placebo and non-ischemic transcriptomic data from the Cook *et al.* study. The placebo ischemic and non-ischemic data was accessed from GEO (accession: GSE35589) and analysed using the methods described above.

Brain-blood gene overlap

Significant overlap between brain and blood differential expression results was determined using the rank-rank hypergeometric overlap test (Plaisier *et al.*, 2010. *Nucleic Acids Res.* **38**(17):e169). This method performs a hypergeometric test on all possible overlaps of the sorted lists of genes in order to identify the cut-off at which the overlap between the two sets are most significant. The full gene lists were sorted by the average log fold change between the two monkeys. In order to examine specific genes whose expression changed in the brain and the blood, we produced a filtered list of highly differentially expressed genes in the brain (see methods above) and calculated the maximum fold change value for each of these genes in the blood.

Gene-set enrichment analysis

Gene set enrichment analysis was performed using the GSEA command line tool (Subramanian *et al.*, **2005**. *Proc Natl Acad Sci U S A.* **102(43)**:15545-50). This tool provides a predefined set of genes and determines whether each set is enriched near the top or bottom of the sorted experimental list, which is indicative of a phenotypic role. GSEA calculates an enrichment score (ES) for each gene set evaluated. This score indicates the degree to which the set is overrepresented at the top or bottom of the sorted experimental gene list. The normalized enrichment score (NES) is normalized by the size of the gene set.

Results from brain and blood differential expression analysis were sorted by log fold change. These lists were passed to GSEA pre-ranked function. The analysis was run using 100 permutations, and gene sets with fewer than 10 genes were excluded. The resulting gene sets were sorted by the NES. The GSEA ES indicates the degree to which a gene set is overrepresented at the top or bottom of the sorted gene list. The ES is calculated by moving down the list and increasing the score when a gene is present and decreasing when it is not. The magnitude by which the score is increased depends on the correlation of the gene with the phenotype (predefined gene set). The ES is the maximum deviation from 0 encountered when moving down the list. A positive ES indicates enrichment at the top of the list (in the upregulated genes), and a negative ES indicated enrichment at the bottom. The normalized ES accounts for differences in the size of the gene set and for correlations between gene sets and the expression data, allowing for comparisons between gene sets.

The interactions between genes in these gene sets were visualized using the STRING database (Szklarczyk *et al.*, **2015**. *Nucleic Acids Res.* **43(Database issue)**:D447-52). Genes were entered into STRING if they had a fold change of at least 2 in the brain, and 1.5 in the blood. The thickness of the lines indicates the confidence that a relationship exists and the large nodes indicate proteins for which there is information about the tertiary structure.

Results

Animals were sacrificed about 5.5 hours after ischemia onset. Axial MRI diffusion weighted image showed an MCA focal ischemia in both animals (**data not shown**). Analysis of the volume of ischemia in the two different animals showed that the volume of infarction was highly variable: S1 had a visibly larger infarction volume than S2. Superficial and deep infarctions were observed for animal S1 and S2. The infarction was also visible on T2 flair image in both animals. Willis Angio-MRI showed that MCA was proximally occluded for animal S1 while it was less clear for animal S2. Infarction was also visible on T1 sequence at the level of basal ganglia in animals S1 and S2.

10 *Gene expression changes: ischemic versus non-ischemic brain tissue*

In this study, changes in expression between ischemic and non-ischemic brain tissue was measured for individual genes, and the effect of these changes was examined at the level of gene sets. A differential expression analysis was performed comparing expression data from ischemic brain tissue to data from the corresponding region of the contralateral hemisphere, which did not undergo ischemia.

This analysis revealed that the expression patterns of ischemic and non-ischemic brain samples are visibly different (**data not shown**). Genes were considered highly differentially expressed if they had a fold change greater than 2 when comparing ischemic and non-ischemic brain samples within each monkey and these genes were not differentially expressed when comparing ischemic tissue between monkeys or non-ischemic tissue between monkeys (fold change < 2). When hierarchical clustering was applied to these top differentially expressed genes (37 genes), the ischemic samples clearly cluster independently from the non-ischemic samples.

All of the highly differentially expressed genes were up-regulated (**Table 1**).

25 **Table 1: the list of genes that were deemed highly differentially expressed in ischemic brain after extensive filtering.**

The values show in columns “S1 brain” and “S2 brain” are the log₂ fold change values of these genes in the brain of the two monkeys.

Columns “S1 max blood”, “S1 timepoint”, “S2 max blood” and “S2 timepoint” show data from the blood of the same two monkeys. The time point with the maximum log₂ fold change is shown for each.

- Genes that are highly differentially expressed in the blood (fold change > |1.5|) appear in bold and greyed out.

HSPA1B and *LOC720054 (HSPA1B)* are replicate probes for different regions of the same gene.

Gene symbol	S1 brain	S2 brain	S1 max blood	S1 timepoint	S2 max blood	S2 timepoint
<i>HSPA1B</i>	4.67	5.40	1.86	T₀ vs T₃	0.99	T₀ vs T₁
<i>LOC720054 (HSPA1B)</i>	4.51	5.25	1.70	T ₀ vs T ₃	0.53	T ₀ vs T ₁
<i>NPAS4</i>	3.57	2.65	0.05	T ₀ vs T ₂	0.06	T ₀ vs T ₂
<i>LOC718890 (DNAJB1)</i>	3.37	4.16	1.12	T ₀ vs T ₁	0.22	T ₀ vs T ₁
<i>ATF3</i>	2.99	3.38	0.27	T ₀ vs T ₁	-0.04	T ₀ vs T ₁
<i>HSPB1</i>	2.85	3.30	-0.63	T ₀ vs T ₂	-0.15	T ₀ vs T ₃
<i>RRAD</i>	2.74	4.26	0.81	T ₀ vs T ₃	-0.03	T ₀ vs T ₁
<i>NR4A1</i>	2.72	2.88	0.03	T ₀ vs T ₁	-0.27	T ₀ vs T ₃
<i>CYR61</i>	2.61	2.34	0.11	T ₀ vs T ₂	0.17	T ₀ vs T ₃
<i>C-FOS</i>	2.53	3.77	0.17	T ₀ vs T ₂	0.45	T ₀ vs T ₂
<i>GADD45G</i>	2.25	2.04	0.44	T ₀ vs T ₃	0.87	T ₀ vs T ₃
<i>RGS1</i>	2.01	2.04	0.23	T ₀ vs T ₁	0.57	T ₀ vs T ₂
<i>LOC714407 (EGR4)</i>	1.84	1.76	-0.19	T ₀ vs T ₂	0.28	T ₀ vs T ₃
<i>ARC</i>	1.84	1.90	0.27	T ₀ vs T ₃	0.08	T ₀ vs T ₃
<i>PTGS2</i>	1.84	1.41	3.09	T₀ vs T₂	3.78	T₀ vs T₃
<i>RGS2</i>	1.81	1.79	0.10	T ₀ vs T ₂	0.81	T ₀ vs T ₂
<i>CCL3</i>	1.78	2.18	-0.49	T ₀ vs T ₁	0.00	T ₀ vs T ₃
<i>BAG3</i>	1.70	2.97	0.99	T₀ vs T₁	0.71	T₀ vs T₁
<i>HSPA4L</i>	1.68	1.64	-0.13	T ₀ vs T ₁	0.30	T ₀ vs T ₃
<i>EGR2</i>	1.68	2.09	-0.12	T ₀ vs T ₂	-0.16	T ₀ vs T ₃
<i>ADM</i>	1.66	2.59	2.60	T₀ vs T₃	0.86	T₀ vs T₃
<i>TM4SF1</i>	1.61	2.42	0.65	T₀ vs T₃	1.01	T₀ vs T₃
<i>EGR1</i>	1.56	2.10	0.22	T ₀ vs T ₁	0.07	T ₀ vs T ₃
<i>DUSP1</i>	1.43	1.34	2.08	T₀ vs T₃	1.87	T₀ vs T₂
<i>BTG2</i>	1.42	1.38	0.39	T ₀ vs T ₁	0.14	T ₀ vs T ₂
<i>LOC715456</i>	1.36	1.14	1.33	T ₀ vs T ₁	0.15	T ₀ vs T ₃
<i>HMOX1</i>	1.30	1.13	2.81	T₀ vs T₃	0.83	T₀ vs T₃

highly similar to human <i>LDLR</i> [CN641580]	1.29	1.12	2.60	T ₀ vs T ₁	1.56	T ₀ vs T ₁
<i>DNAJA4</i>	1.28	1.65	0.56	T ₀ vs T ₃	-0.03	T ₀ vs T ₁
<i>MCL1</i>	1.24	1.52	0.56	T ₀ vs T ₃	0.34	T ₀ vs T ₁
<i>LOC720001</i> (<i>HSPA6</i>)	1.20	1.07	0.31	T ₀ vs T ₁	0.08	T ₀ vs T ₁
<i>GADD45B</i>	1.08	2.17	0.46	T ₀ vs T ₂	0.94	T ₀ vs T ₂
<i>IL6</i>	1.08	1.53	-0.47	T ₀ vs T ₃	-0.10	T ₀ vs T ₂
<i>ADFP</i>	1.08	1.25	0.61	T ₀ vs T ₁	0.34	T ₀ vs T ₁
<i>HES4</i>	1.08	1.18	-0.04	T ₀ vs T ₁	0.13	T ₀ vs T ₁
<i>DUSP5</i>	1.05	1.67	-0.30	T ₀ vs T ₁	-0.78	T ₀ vs T ₁
<i>GEM</i>	1.04	1.55	0.39	T ₀ vs T ₂	0.19	T ₀ vs T ₁
<i>LOC717581</i> (similar to <i>G0S2</i>)	1.00	1.96	1.90	T ₀ vs T ₂	1.47	T ₀ vs T ₃

Internal validation

To confirm the robustness of the microarray results, mRNA levels of three ischemic-sensitive genes (*HSPA1B*, *GADD45G*, *CDKN1A*) were quantified by RT-qPCR using samples from the animals. *SMC2*, which was found to exhibit invariant expression level across all test samples, was used as internal reference (housekeeping). Three reverse transcriptions were performed for each RNA samples, followed by three independent qPCR runs, with replicate assay measurements for both target and reference genes. For all genes, the direction and magnitude of the change agreed well with the microarray data (Figure 1 and Table 2).

10 Table 2: internal validation.

Statistical significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Results were normalized to *SMC2*.

Gene	Microarray study		qPCR validation	
	Fold change	<i>p</i> -value	Fold change	<i>p</i> -value
<i>HSPA1B</i>	32.78	3.80×10^{-18}	33.3	***
<i>GADD45G</i>	4.41	9.40×10^{-10}	3.7	**
<i>CDKN1A</i>	2.05	1.49×10^{-4}	2.3	**

External validation

Analysis of transcriptome data from the Cook *et al.* study of stroke (2012. *Nature*. **483(7388)**:213-7) in *Macaca fascicularis* produced very different results than what has been described here. Whereas this analysis of *Macaca mulatta* revealed many up-regulated genes in the tissues affected by stroke, the data from *Macaca fascicularis* showed mainly down-regulated genes. This is mostly likely due to the very different method for inducing stroke in the primates. The study by Cook *et al.* used surgical middle cerebral artery occlusion (MCAO), which they acknowledged produces a more severe stroke than is usually observed in human. It is likely that this more severe stroke model caused massive cell death resulting in mainly down-regulated genes.

Gene sets enrichment

Gene sets that are enriched in ischemic brain tissue were identified using the Broad Institutes Gene Set Enrichment Analysis (GSEA) (Subramanian *et al.*, 2005. *Proc Natl Acad Sci U S A*. **102(43)**:15545-50). The top 5 enriched gene sets from GSEA's Hallmark gene sets were TNFA signalling via NFKB, apoptosis, P53 pathway, hypoxia, and UV response up, all of which were up-regulated (Table 3).

Table 3: the top 5 gene sets resulting from GSEA analysis differential expression results in the brain.

Gene Set Name	Set size	Genes present (percentage)	NES
TNFA signalling via NFKB	108	60 (55.56%)	2.9935637
Apoptosis	98	26 (26.53%)	2.5899184
P53 pathway	112	28 (25%)	2.5179965
Hypoxia	118	33 (27.97%)	2.455276
UV response up	96	31 (32.29%)	2.3801901

60 out of the set of 108 genes had a core enrichment in the TNFA signalling via NFKB gene set (NES = 2.96). Almost all genes in this set for which we have data are up-regulated, none are significantly down-regulated genes: 15 genes only are slightly down-regulated with a maximum fold change of 1.17.

In the apoptosis gene set, 26 out of 98 genes have core enrichment (NES = 2.57).

In the hypoxia gene set, 33 out of 118 genes had core enrichment (NES = 2.38).

The UV response up gene set had 31 out of 96 genes with core enrichment (NES = 2.3).

The most significantly enriched down-regulated gene set was oxidative phosphorylation.

5 84 of these genes, out of 120, had core enrichment (NES = -2.57).

Interaction of gene products

The STRING database (Szklarczyk *et al.*, 2015. *Nucleic Acids Res.* **43(Database issue):D447-52**) was used in order to visualize the interaction of the gene products of genes that are highly differentially expressed in the brain and blood within the top gene sets of the brain (fold change ≥ 2 in the brain, fold change ≥ 1.5 in the blood) (**data not shown**).

It was noted that IL-6 is a highly connected member in the networks representing TNFA signalling via NFkB, apoptosis, and hypoxia. Additionally, *CDKN1A* appears in all four of these networks. The interaction of *CDKN1A* with *HMOX1* is observed in the network of apoptosis-, p53 pathway-, and hypoxia-related genes. The gene *ATF3* also appears in all four networks, and it interacts with IL-6 in the three mentioned above. The 5th ranking gene set in the brain (**Table 3**), UV response up, is not shown because none of the top differentially expressed genes in this set have any known interactions.

Gene expression changes: pre-occlusion versus post-occlusion blood samples

20 Differentially expressed genes in the blood during cerebral ischemia were identified by comparing all pre-occlusion blood samples to all post-occlusion blood samples. When hierarchical clustering was applied to the top differentially expressed genes, pre-occlusion and post-occlusion samples cluster separately (**data not shown**). There also appears to be differing expression patterns in the blood of the two macaques based on this gene clustering. Monkey S1 has a more profound upregulation of these genes than monkey S2. This separation of the two monkeys is not apparent in the expression patterns of the brain (**data not shown**).

As with the brain samples, gene set enrichment of the blood differential expression results was analysed. The top 5 enriched gene sets are TNFA signalling via NFkB, hypoxia, hedgehog signalling, inflammatory response, and angiogenesis (**Table 4**).

Table 4: the top 5 gene sets resulting from GSEA analysis differential expression results in the blood.

Gene set name	Set size	Genes present (percentage)	NES
TNFA signalling via NFkB	108	37 (34.36%)	1.9190117
Hypoxia	118	42 (25.59%)	1.9131837
Hedgehog signalling	24	10 (41.67%)	1.779471
Inflammatory response	110	35 (31.82%)	1.7678385
Angiogenesis	20	9 (45%)	1.7562431

The TNFA signalling via NFkB and hypoxia gene sets also appeared in the top 5 gene sets for the brain (**Table 4**).

37 out of 108 genes in the TNFA signalling via NFkB gene set had core enrichment (NES = 1.92).

42 out of 118 genes in the hypoxia gene set had core enrichment (NES = 1.91).

10 out of 24 genes in the hedgehog signalling gene set had a core enrichment (NES = 1.77).

35 out of 110 genes in the inflammatory response gene set had core enrichment (NES = 1.77).

9 out of 20 genes in the angiogenesis gene set had core enrichment (NES = 1.76).

Differentially expressed genes common between the brain and the blood

Differential expression results from the brain and blood were examined for significant overlap when genes were sorted by fold change. We show that the two sorted gene sets are highly similar, particularly at the two extremes: the most upregulated and most downregulated genes during ischemia. We also showed the overlapping genes from the

heatmap that are most significant: 2156 down regulated genes significantly overlap between brain and blood samples, and 493 up-regulated genes significantly overlap (**Figure 2**). The majority of these genes have a relatively low expression fold-change, however the overlap of differentially expressed genes is very high between brain and
5 blood samples.

Although many genes in these overlapping sets were not highly differentially expressed, some were, and it is those with a very high change in expression in the brain that we were most interested in. Of the genes identified as highly differentially expressed in the brain, 9 were also differentially expressed in the blood (fold change ≥ 1.5 ; **Table 1**, highlighted
10 in bold). All 9 of these genes occur within the most significant hypergeometric overlap (bolded). These genes are *PTGS2*, *G0S2*-like, *DUSP1*, *LDLR*-like, *HMOX1*, *HSPA1B*, *BAG3*, *ADM*, and *TM4SF1*. The majority of these genes have a sharp increase in expression in the blood, which levels off over time (**Figures 3 and 4**).

Discussion

15 A non-human primate model (Gauberti *et al.*, **2012**. *Cerebrovasc Dis.* **33**(4):329-39) with human thrombin injection in the MCA artery was used to study patterns of gene expression changes during ischemic cerebral stroke. This is an embolic model of focal ischemia with a partial MCA ischemia. A time frame of 6 hours was used, because this is the window of time where therapeutic interventions are possible in human (thrombolysis
20 up to 4h30; thrombectomy up to 6h after onset).

Our study of macaque microarray expression data from brain and blood revealed that ischemic and non-ischemic samples can be distinguished based on their expression profiles, and the majority of highly differentially expressed genes are up-regulated in the ischemic scenario 6 hours after ischemia. Many of these up-regulated genes belong to
25 pathways involved in cell death and DNA damage repair. A comparison of genes differentially expressed in the brain and the blood revealed a significant overlap of gene expression patterns.

These results indicate the potential to identify ischemic stroke through transcriptomics in the brain and the blood.

Gene expression in ischemic brain

Quality of the data was confirmed by PCA analysis showing that it was possible to differentiate both monkeys and ischemia from non-ischemia by gene expression. Furthermore, internal validation further supported generated results.

- 5 Hierarchical clustering of *Macaca mulatta* brain expression data revealed strong independent clustering of ischemic and non-ischemic brain samples (**data not shown**). These results indicated a potential for identifying ischemic brain tissue based on expression profiles. The most significantly differentially expressed genes were up-regulated, which is consistent with many previous studies of ischemia in mouse (Büttner *et al.*, **2009**. *Brain Res.* **1252**:1-14; Hori *et al.*, **2012**. *Dis Model Mech.* **5**(2):270-83). This
- 10 study defined highly differentially expressed genes in the brain as genes that have a minimum fold change of 2 in both monkeys, and the genes are not differentially expressed when comparing non-ischemic samples between monkeys or when comparing ischemic samples between monkeys (fold change < 2). The results of our analysis found that 37
- 15 genes were up-regulated and none were down-regulated in the brain. These results are similar to those of Büttner *et al.* which found 115 up-regulated and 19 down-regulated genes after 6 hours (Büttner *et al.*, **2009**. *Brain Res.* **1252**:1-14). The larger number of genes identified by Büttner *et al.* can be explained by the larger expression profile array used and expression differences between species.
- 20 All highly differentially expressed genes in ischemic brain tissue were up-regulated. Many of these genes have been previously identified as up-regulated genes in ischemic stroke for a variety of functions, notably stress response, apoptosis, and signal transduction.

- 25 Among the up-regulated stress response genes were 7 heat shock proteins *HSPA1B* (Hsp70), *HSP40*, *HSPB1*, *HSPA4L*, and *DNAJA4* (Hsp40-like) (**Table 1**). Numerous studies have identified heat shock proteins as highly up-regulated in ischemia (Schmidt-Kastner *et al.*, **2002**. *Brain Res Mol Brain Res.* **108**(1-2):81-93; Büttner *et al.*, **2009**. *Brain Res.* **1252**:1-14; Kawahara *et al.*, **2004**. *J Cereb Blood Flow Metab.* **24**(2):212-23; Tang *et al.*, **2002**. *Eur J Neurosci.* **15**(12):1937-52). The human ortholog of this gene is well

characterized as a stress-induced gene that stabilizes proteins against aggregation, and is involved in the ubiquitin-proteasome pathway (NCBI, Accession: NM_005346.5). Hsp70 is the most commonly reported up-regulated heat shock protein in ischemic stroke (Cox-Limpens *et al.*, **2014**. *Brain Res.* **1564**:85-100). In *Macaca mulatta*, Heat Shock Protein Family A (Hsp70) Member 1B (*HSPA1B*) is the most up-regulated gene in ischemic brain tissue. Knockout studies of *HSPA1B* in mice showed that cardiac cells lacking this gene are more susceptible to damage by ischemia (Kim *et al.*, **2006**. *Circulation.* **113**(22):2589-97). Studies of cerebral ischemia showed hsp70 knockout mice experienced a greater infarction volume than wild type mice (Lee *et al.*, **2004**. *Stroke.* **35**(9):2195-9), and mice that over-express Hsp70 exhibited a decreased infarct size and improved neurological function (Zheng *et al.*, **2008**. *J Cereb Blood Flow Metab.* **28**(1):53-63). The high level of differential expression of *HSPA1B* in macaque indicates it may also be playing a neuro-protective role in primate brains undergoing ischemia. It is also interesting to note that one of the most differentially expressed gene from these microarrays is *DNAJB1*, a heat shock protein homologue.

NPAS4 is another highly differentially expressed stress response gene that is also known to be a neuroprotectant (Choy *et al.*, **2015**. *Int J Mol Sci.* **16**(12):29011-28). This transcription factor is expressed in the neurons of the brain, and plays a role in early response in excitatory and inhibitory neurons. Its expression is induced by a variety of situations that put neuronal cells under stress. In our study, *NPAS4* had a log₂ fold change of 3.57 in S1 and 2.65 in S2 (**Table 1**). The gene *C-FOS*, which is controlled by *NPAS4* (Ramamoorthi *et al.*, **2011**. *Science.* **334**(6063):1669-75), is also highly differentially expressed in our study with a log₂ fold change of 2.53 in S1 and 3.77 in S2. *C-FOS* is commonly found to be up-regulated in the presence of cerebral ischemia (Cox-Limpens *et al.*, **2014**. *Brain Res.* **1564**:85-100).

The gene Activating Transcription Factor 3 (*ATF3*) is the next most differentially expressed gene. *ATF3* encodes a member of the cAMP responsive element-binding (CREB) family of transcription factors. It is involved in cellular stress response. *ATF3* underwent a log₂ fold change of 2.99 in S1 and 3.38 in S2 (**Table 1**). Additionally, this gene is a highly connected member within the top GSEA gene sets (**data not shown**).

- Previous experiments on mice found that *ATF3* knockouts have a larger infarction volume and worsened neurological function after brain ischemia (Wang *et al.*, **2012**. *Neuroscience*. **220**:100-8).
- Other important stress response genes which were upregulated in ischemic brain tissue
5 are two damage-inducible genes, *GADD45G* and *GADD45B* (Growth Arrest and DNA-Damage inducible 45). *GADD45* genes have been implicated in DNA repair. *GADD45G* underwent a log₂ expression fold change of 2.25 in S1 and 2.04 in S2, for *GADD45B* this was 1.08 and 2.17, respectively. Like heat shock proteins the Gadd45 family of genes have been reported as up-regulated by a number of rodent studies examining cerebral
10 ischemia (Schmidt-Kastner *et al.*, **2002**. *Brain Res Mol Brain Res*. **108(1-2)**:81-93; 7–9, 20). A previous analysis of rat brain during ischemia revealed that expression of these genes is induced by transient global ischemia (Chen *et al.*, **1998**. *J Cereb Blood Flow Metab*. **18(6)**:646-57). These researchers concluded that this could indicate a neuroprotective role for these genes. Our results show this increased expression during
15 ischemia also exists in primates, further implicating its role in ischemic cellular response.
- The apoptotic gene *BAG3* was also significantly upregulated in ischemic brain tissue, with a log₂ fold change of 1.7 in S1 and 2.97 in S2. An expression study of cerebral ischemia in rats also reported this gene as highly upregulated (Schmidt-Kastner *et al.*, **2002**. *Brain Res Mol Brain Res*. **108(1-2)**:81-93). The dissolution of cytoskeleton proteins
20 is known to occur after ischemia (Lipton, **1999**. *Physiol Rev*. **79(4)**:1431-568), and interestingly, we observed that the gene activity-regulated cytoskeleton-associated protein (*ARC*) is up-regulated in ischemic brain tissue (log₂ fold change of 1.84 in S1 and 1.9 in S2). Up-regulation of *ARC* has been observed in previous studies of cerebral ischemia in rat (Büttner *et al.*, **2009**. *Brain Res*. **1252**:1-14).
- 25 Another class of genes that have been implicated in cerebral ischemia is dual-specific phosphatases (Dusp) (Wang *et al.*, **2011**. *Brain Res*. **1372**:13-21). Their protein products are able to inactivate MAPK proteins. Two Dusp genes were highly up-regulated in our study, *DUSP1* (log₂ fold change of 1.43 in S1 and 1.43 in S2) and *DUSP5* (log₂ fold change of 1.05 in S1 and 1.67 in S2). Interestingly, Dusp5 has been suggested to be a
30 target of p53 (Ueda *et al.*, **2003**. *Oncogene*. **22(36)**:5586-91).

Gene expression changes in the blood

The expression patterns observed in the blood were similar to that of the brain, however there were some limitations in this study regarding the blood transcriptomics. Due to the relatively small number of samples (1 sample per time point for each monkey), time course analysis was not feasible. In lieu of a time course analysis, samples collected before occlusion were compared to each post-occlusion sample in a pairwise manner. In order to perform gene set analysis, the differential expression results from the comparison that yielded the largest change in expression was used with GSEA. The gene sets implicated by this analysis showed a strong correlation with expression patterns in the brain.

Hierarchical clustering of blood expression levels revealed that pre- and post-occlusion samples cluster independently (**data not shown**). Although the separation is not as stark as it is for the brain samples, there is still a clear difference in gene expression between pre- and post-occlusion. These results suggest that further experiments could reveal a panel of blood biomarkers which may be used for the diagnosis of stroke. Due to the small number of samples and individuals in this study, it would be difficult to specify a subset of genes that may be used for diagnosis, however the distinct expression pattern suggests that with a larger sample size, this would be possible.

Our analysis of differentially expressed genes in the blood of macaques undergoing cerebral ischemia revealed that, like the brain, the majority of transcripts in the blood are up-regulated. When samples are pooled between monkeys, and all post-occlusion samples are pooled, 651 genes have a high level of differential expression (fold change more than 2), out of which 513 are up-regulated.

Notable among these differentially expressed genes are four S100 genes: *S100A8*, *S100A12*, *S100P*, and *S100A9*. S100 are calcium-binding proteins from glial cells. The most highly differentially expressed of this group is *S100A8* ($\log_2$ fold change of 3.29, $p = 0.027$; **data not shown**), which is strongly associated with pro-inflammatory functions (Sedaghat & Notopoulos, 2008. *Hippokratia*. **12(4)**:198-204).

Gene sets implicated in cerebral ischemia

Brain

Gene set enrichment analysis of brain expression data revealed several gene sets involved in DNA repair and apoptosis that are up-regulated in the ischemic brain samples. The top
 5 5 enriched gene sets were TNFA signalling via NFkB, apoptosis, p53 pathway, hypoxia, and UV response up. Some of these pathways have been described as related to cerebral ischemia in previous studies.

The most enriched gene set in the brain is tumor necrosis factor alpha (TNFA) signalling via NF-κB. The TNFA cytokine activates NFkB, which is involved in inflammatory
 10 response. A number of previous studies have reported up-regulation of pro-inflammatory cytokines (Schmidt-Kastner *et al.*, **2002**. *Brain Res Mol Brain Res*. **108(1-2)**:81-93; Lu *et al.*, **2004**. *J Neurosci Res*. **77(6)**:843-57; Broughton *et al.*, **2009**. *Stroke*. **40(5)**:e331-9).

Also, up-regulated were gene sets involved in apoptosis and the p53 pathway. These pathways are activated in the event of DNA damage or cell stress, and previous studies
 15 have noted up-regulation of various pro-apoptotic factors (Büttner *et al.*, **2009**. *Brain Res*. **1252**:1-14).

Additionally, the pathways of NFkB and p53 have been shown to contribute to neuroprotection (Zhang *et al.*, **2005**. *J Cereb Blood Flow Metab*. **25(1)**:30-40; Chen *et al.*, **2011**. *Antioxid Redox Signal*. **14(8)**:1505-17).

20 It is interesting to note that the gene IL-6 is a highly connected member of the TNFA signalling, apoptosis, and hypoxia pathways (**data not shown**). This gene has a log₂ fold increase in expression of 1.08 and 1.53 in S1 and S2 brain samples, respectively. The *HMOX1-CDKN1A* interaction is also common across several gene sets (apoptosis, p53, and hypoxia), and the up-regulation of *HMOX1* is also significant.

25 Blood

Gene set enrichment analysis of the differential expression results from the blood revealed pathways involved in signalling, hypoxia, and inflammatory response. The top 5 gene

sets enriched ischemic blood samples were TNFA signalling via NFkB, hypoxia, hedgehog signalling, inflammatory response, and angiogenesis.

Interestingly, two of the most enriched gene sets in brain are also enriched in the blood: TNFA signalling via NFkB and hypoxia response. Both of these pathways fit well into the model of ischemia response. 37 out of the 108 genes in the TNFA signalling via NFkB gene set have core enrichment in the blood (NES = 1.92), and 42 out of 118 genes in the hypoxia response gene set have core enrichment (NES = 1.91). The gene set TNFA signalling via NFkB appears to be the pathway most involved in ischemia, with over 50% of the set having core enrichment in brain and over 30% in the blood.

10 *Common expression between brain and blood*

In order to compare brain and blood differential expression, all genes were sorted by fold change and every possible hypergeometric overlap was tested, which revealed that there was a significant overlap of both up- and down-regulated genes in the gene lists. Our analysis of the most significant hypergeometric overlap of up- and down-regulated genes revealed that the intersections contains 493 and 2156 genes, respectively (**Figure 2**).

An additional, more stringent, analysis of blood expression data was also performed where pre- and post-occlusion samples in each monkey were compared separately, and only the time point resulting in the largest fold change was kept. These results were merged with the brain expression data and genes were filtered for high differential expression in both brain (fold change ≥ 2) and blood (fold change ≥ 1.5). 9 genes were identified as highly differentially expressed in both the brain and the blood (**Table 1**). All 9 of these genes appear in the most significant hypergeometric overlap described above.

These top 9 genes are *HSPA1B*, *PTGS2*, *BAG3*, *ADM*, *TM4SF1*, *DUSP1*, *HMOX1*, *LDLR*-like, and *GOS2*. *HSPA1B*, *BAG3*, and *DUSP1* have been discussed in previous sections. The majority of these genes have a sharp increase in expression, which levels off over time (**Figures 3 and 4**).

Prostaglandin-Endoperoxide Synthase 2 (*PTGS2/COX2*) is an enzyme that plays a role in prostaglandin biosynthesis. This gene is known to become up-regulated during

inflammation, and is a target of aspirin. Aspirin suppresses the production of prostaglandins and some dosages reduce the risk of stroke (Tohgi *et al.*, **1992**. *Stroke*. **23(10)**:1400-3; Eikelboom *et al.*, **2002**. *Circulation*. **105(14)**:1650-5).

Adrenomedullin (*ADM*) gene expression levels in the blood have been shown to be
5 associated with the severity of ischemic stroke (Liu *et al.*, **2014**. *Int J Neurosci*. **124(4)**:271-80). Liu *et al.* suggests that *ADM* expression levels in peripheral blood leukocytes could indicate the severity of tissue damage. This hypothesis is also implicated in our results as monkey S1 has a larger infarction volume, as shown by the MRI (**data not shown**), and it also has a higher level of expression of *ADM* in the blood than S2.
10 Although this expression pattern is inverted in the brain.

Heme oxygenase 1 (*HMOX1*) is a member of the heat shock family of proteins we have discussed previously. It is believed to be part of the cellular defence system for oxidative stress-mediated injury, like stroke (Chen & Maines, **2000**. *Cell Mol Biol (Noisy-le-grand)*. **46(3)**:609-17). Additionally, previous studies have found *HMOX1* to be up-
15 regulated in the brain after cerebral ischemia (Zhao *et al.*, **2017**. *J Stroke Cerebrovasc Dis*. **26(7)**:1622-1634).

Conclusions

Our data showed that there is a common signature between the ischemic brain and the blood, and support the development of blood transcriptomics as a tool for biopsy
20 transcriptome expression profiling, to characterize patients with ischemic stroke and/or to develop a companion biomarker for the assessment of neuroprotection drugs in patients with ischemic stroke.

Although it is known that plasma extracellular noncoding RNAs (ex-RNAs) are a class of circulating RNA molecules that directly modulate networks of gene expression in
25 target tissues (Mick *et al.*, **2017**. *Stroke*. **48(4)**:828-834), it was quite unexpected to identify common profile of RNA coding sequence in the brain and blood. This pattern of expression of ischemia-related genes being observed in the blood of patients with cerebral ischemia has been noted before in study by Moore *et al.* (**2005**. *Circulation*. **111(2)**:212-21). They found genes related to hypoxia in PBMC even though the PBMC itself was not

hypoxic, though the brain was. Our results further Moore *et al.*'s conclusion that blood could be used as a diagnostic tool for confirming ischemic stroke.

Example 2: Clinical study protocol

We used a non-human primate model (Gauberti *et al.*, **2012**, *Cerebrovasc Dis.* **33(4)**:329-39), with human thrombin injection in the MCA artery. Our study revealed that ischemic and non-ischemic samples can be distinguished based on their expression profiles 6 hours after ischemia. Identified upregulated genes belong to pathways involved in cell death and DNA damage repair. Comparison of genes differentially expressed in the brain and the blood revealed a significant overlap of gene expression patterns.

Together, these results indicated the potential to identify ischemic stroke through transcriptomic profile in the brain and the blood.

Nine most significantly overlapping up-regulated genes in brain and blood were identified and are used further in a human study.

We propose to translate the data generated from monkeys to humans. Up-regulated genes identified both in ischemic brain and in peripheral blood in the primate model of ischemic stroke are studied in samples from 20 ischemic patients, 20 haemorrhagic patients and matched controls. We then look to the corresponding proteins in order to be able to potentially develop a rapid detection test as already performed in other cardiovascular diseases.

Pilot case-control study

Case-control study with cases being first ever ischemic stroke paired for age, sex and cardiovascular risk measured by a score (European Heart Score) before the index stroke in patients.

Ischemic cases are recruited from the Brest stroke registry. The registry includes 900 patients per year. The control group is recruited from one centre (Brest University hospital).

Primary objective:

To determine an RNA blood biomarker test based on genes identified whose expression is significantly increased.

Design of the study:

- 5 Case-control study with case being first ever ischemic stroke paired for age, sex and cardiovascular risk measured by a score (European Heart Score) before the index stroke in patients.

Primary endpoint:

Expression (measured in $\log_2$) of each of the genes identified 6 hours after ischemia onset.

10 ***Primary statistical analysis:***

The expression of each candidate genes is compared in case and controls with a student test for paired data.

Secondary statistical analysis:

- 15 The expression of each gene is compared for cases between the acute phase of ischemic stroke and 3 months after.

Sample size estimation for the full study:

The difference between case and controls is set at 1.5-fold for cases compared to controls (Fold Change $FC = 1.5$ and $\log_2 FC = 0.585$), for a standard deviation of $\log_2$ expression intensity inferior to 1 (personal data).

- 20 With these hypotheses (difference = 0.585 and standard deviation = 1), a power of 90% and a Bonferroni correction for tests multiplicity, the estimation of the number of required subjects is 110 cases and 110 controls. This calculation does not take in account the effect of the possible increase of power provided by pairing, since the correlation level of the response is unknown.

Inclusion criteria***For patients:***

Age > 18-year-old; carotid ischemic stroke onset inferior to 6 hours; ability to have the T₁ blood sample before 6 hours; eligibility to thrombolysis or thrombectomy.

- 5 Initial NIHSS score > 0; patients with CT or MRI imaging compatible with an ischemic stroke; patients with multimodal imaging allowing to analyse penumbra either through MRI or CT perfusion and vessels supra-aortic vessels and intra-cerebral vessels (Willis circle) imaging.

Exclusion criteria includes patients with intracerebral haemorrhage.

10 ***For controls:***

Age > 18-year-old; stroke-free standardized questionnaire; high risk cardiovascular subjects without cardiovascular disease and no stroke.

Case Report Form (CRF):

- 15 For each patient and control, demographic and clinical characteristics, cardiovascular risk factors and current treatment are recorded using the standardized report form of the Brest Stroke registry.

Cerebral imaging:

At the acute phase, perfusion MRI or CT and angio-MR or angio-scan

Clinical and imaging follow-up:

- 20 For patients, rankin scores at 3 months $\pm$ 15 days and cerebral MRI at 3 months $\pm$ 15 days.

Number of samples per patients:

42 mL of blood are drawn from 6 different times: < 6 hours, 12 hours $\pm$ 2, 24 hours $\pm$ 4, 48 hours $\pm$ 4, 7 days $\pm$ 2 days, and at 3 months $\pm$ 15 days after stroke onset.

Number of samples per control:

42 mL of blood are drawn once for each control.

Number of patients and controls:

- 20 patients for ischemic cases; 20 controls matched with ischemic cases for age, sex and
5 European Heart score.

Hemorrhagic patients

The haemorrhagic cases are recruited from the Brest stroke registry as a different group with the same number of samples per patient as in the ischemic group.

Inclusion criteria

- 10 *For patients:*

Age > 18-year-old; intracerebral haemorrhage with onset inferior to 6 hours; ability to have the T₁ blood sample before 6 hours.

Initial NIHSS score > 0; patients with CT or MRI imaging compatible with a haemorrhagic stroke.

- 15 Exclusion criteria includes patients with traumatic intracerebral haemorrhage and patients with ischemic stroke.

Number of patients

20 patients for haemorrhagic cases.

Ethical aspects:

- 20 The project is conducted in accordance to the Declaration of Helsinki principles and Good Clinical Practice (GCP - ICH E6). It is submitted for approval to a Human Research Ethics Committee in accordance with French regulation for clinical trial. Written informed consent is collected from each participant.

Data management and Biobanking:

Data entry is performed by data manager into a database managed by the software Clinsight (Capture system). The biobank is stored in Brest University Hospital's centre for biological resources (CRB – Centre de Ressources Biologiques) certified NFS 96-900.

Recruitment of patients and controls***Recruitment Centers:***

Patients and control phenotype as well as blood collection are implemented via the CHRU of Brest.

10 Imaging characterization and centralized post-processing of data

The aim of the initial functional imaging (CT or MRI perfusion) is to map the core of the infarction and its penumbra, and to follow the evolution of both zones at 3 months on MRI. The initial imaging takes into account vessel patency. A software allows a standardized perfusion analysis for MRI and CT scan. Another software allows the estimation of final volume on MRI and the comparison with the penumbra evaluated on the first imaging.

Imaging platforms:

A 64-row (or more) multi-detector CT scan or a 1.5 Tesla MRI or 3.0 Tesla MRI scanners is used with contrast media for initial imaging.

20 *Methods of data acquisition:*

Either of:

- CT non-contrast head; CT brain perfusion; Computed Tomography Angiography (CTA) head and neck; or
- Brain MR including Diffusion-Weighted Imaging (DWI), FLuid Attenuated Inversion Recovery (FLAIR), and Gradient Recalled Echo (GRE), Magnetic Resonance Angiography (MRA) of the head using Time Of Flight, MR brain

perfusion using Dynamic Susceptibility Contrast (DSC), Gadolinium MRA of the aortic arch and cervical arteries.

In the CT group, the ischemic core is evaluated either by relative cerebral blood flow or cerebral blood volume (CBV). In the MR group of patients, the ischemic core (tissue
5 considered irreversibly injured) is evaluated based on an apparent diffusion coefficient (ADC) threshold.

Critically hypoperfused tissue is evaluated for MR and CT group based on time to maximum signal of a deconvolved signal intensity curve (T_{max}) threshold of > 6 seconds. The DWI lesion volume or CT Alberta Stroke Program Early CT Score (ASPECTS) is
10 recorded for MRI or CT, respectively. The location of the occlusion (internal carotid artery (ICA), M1, M2, basilar, or no occlusion) is recorded.

At 3 months, brain MR including DWI, 3D T2-FLAIR and 3D T1 Gradient Echo, T2(GRE), MRA of the head using TOF.

Post-imaging processing

15 Raw DICOM data of perfusion imaging (CT and MRI) are post-processed using perfusion mismatch analyser software. The final infarct volume is measured on the T2 FLAIR according to segmentation methods. Engineers from the Laboratory of Medical Information Processing (LaTIM - INSERM UMR 1101 Brest) participate to the study.