Current and future bioanalytical approaches for stroke assessment

Article in Bioanalysis - May 2015
DOI: 10.4155/bio.15.40 - Source: PubMed

CITATIONS 3
READS 425

4 authors, including:

Swathi Reddy Pullagurla
University of North Carolina at Chapel Hill
10 PUBLICATIONS 132 CITATIONS

Mateusz Adamski
Jagiellonian University
31 PUBLICATIONS 133 CITATIONS

Steven Soper
Louisiana State University
211 PUBLICATIONS 7,006 CITATIONS

All content following this page was uploaded by Mateusz Adamski on 24 June 2015.
The user has requested enhancement of the downloaded file.
Efforts are underway to develop novel platforms for stroke diagnosis to meet the criteria for effective treatment within the narrow time window mandated by the FDA-approved therapeutic (<3 h). Blood-based biomarkers could be used for rapid stroke diagnosis and coupled with new analytical tools, could serve as an attractive platform for managing stroke-related diseases. In this review, we will discuss the physiological processes associated with stroke and current diagnostic tools as well as their associated shortcomings. We will then review information on blood-based biomarkers and various detection technologies. In particular, point of care testing that permits small blood volumes required for the analysis and rapid turn-around time measurements of multiple markers will be presented.

Incidence & classification of stroke
The World Health Organization (WHO) defined clinical stroke syndrome as ‘rapidly developing clinical signs of focal (or global) disturbance of cerebral function lasting more than 24 h or leading to death with no apparent cause other than that of vascular origin’ [1]. However, substantial improvements in our understanding of anatomy, neuropathology and modern neuroimaging methods has led to the updated definition of stroke. In 2009, the Stroke Council of the American Heart Association (AHA)/American Stroke Association (ASA) redefined, ‘An episode of acute neurological dysfunction presumed to be caused by ischemia or hemorrhage, persisting greater than or equal to 24 h or until death but without sufficient evidence to be classified as any of the other etiologies’ [2].

Stroke is a major cause of neurological disability and mortality affecting more than 15 million people worldwide. In the USA, it is the third leading cause of death after coronary heart disease and all cancers combined, with 795,000 cases reported every year [3,4]. While the stroke mortality rate is decreasing, costs related to stroke will increase due to the ageing population [3]. Total (direct and indirect) annual costs of stroke for 2013 were estimated to be about $72.7 billion, indicating that stroke is a major economic burden on the current healthcare system in the USA [6]. Modifiable risk factors include high blood pressure, diabetes, cardiac diseases, high cholesterol, cigarette smoking, alcohol consumption, obesity, sedentary lifestyle and unhealthy dietary habits [7]. Whereas age, gender, ethnicity and genetics are established as non-modifiable risk factors, better understanding of these risk factors would lessen the global impact of stroke.

Stroke is a heterogeneous disease, which can be classified into two types, ischemic and hemorrhagic (see Figure 1). Both forms result in neurological deficits such as impairment of language, motor function, cognitive decline and vision. Ischemic stroke is caused by interruption of blood flow to the brain parenchyma resulting in a temporary or permanent change in one or more regions of the brain, whereas hemorrhagic stroke is due to the rupture of the cerebral vasculature. The functional changes associated with or resulting from both types are different and complex depending on the nature of the lesion (area of damaged brain tissue).
Ischemic stroke

Ischemic stroke (IS) accounts for 84% of all stroke cases [9]. IS can be further classified into five subtypes based on the causative approach according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST). This approach is globally accepted and in use in a variety of clinical trials. These subtypes include large-artery atherosclerosis, cardio embolism, small vessel occlusion (lacune), stroke of other determined cause and stroke of undetermined cause (cryptogenic stroke) [10].

During ischemia, brain damage or neuronal death is caused by the failure of energy producing compounds like ATP, thereby causing a lack of glucose and oxygen to the brain. The inadequate energy production also affects the functioning of the ion gradient by the loss of potassium ions causing cytotoxic edema (neuronal swelling). Numerous other complex mechanisms are involved in the brain tissue causing IS (see Figure 2A). They include the release of glutamate and aspartate neurotransmitters in the brain, calcium channel dysfunction and production of reactive oxygen species or oxygen radicals activating proteases and lipases that damage cellular and extracellular elements [11,12]. All of these processes result in either the immediate death of a part of the brain parenchyma (core) or partial injury (penumbra; see Figure 2B) with the potential of recovery and therapeutic intervention (see Figure 2C). The duration, severity and location of ischemia are the main factors, which determine the extent of brain damage.

Hemorrhagic stroke

Hemorrhagic stroke (HS) is responsible for about 16% of all stroke cases [14]. Hemorrhage due to a ruptured cerebral blood vessel can be broadly divided into two subtypes: intracerebral hemorrhage (ICH) or subarachnoid hemorrhage (SAH) [3]. ICH originates from the nontraumatic rupture of weakened cerebral vessels; most commonly, small arteries or arterioles causing blood leakage within the parenchymal cerebral space, which forms a localized hematoma (cavity filled with blood). The primary cause of nontraumatic ICH can be attributed to hypertension in 60% of the cases. Other causes include thrombolytic therapy, cerebral amyloid angiopathy, vascular malformations and tumors. SAH, on the other hand, is predominantly triggered by bleeding of the ruptured arteries into the subarachnoid space leaking into cerebral spinal fluid (CSF) blocking its circulation. In addition to this, less common factors include venous infarctions and central nervous system infections [15]. This causes extensive damage to brain cells and therefore, SAH is the most deleterious type of stroke. A report from Andersen et al. demonstrated the higher severity of stroke and mortality rates associated with HS (49.2%) when compared with IS (25.9%) [16].

Stroke mimics & transient ischemic attacks

Stroke mimics (SMs) are defined as diseases caused by neurologic symptoms resembling a stroke with their frequency varying from 1.2 to 25% [17,18] depending on the clinical evaluation. They are reversible with no significant brain damage. The most common type of SMs are neurologic conditions like migraine [19], demyelinating disease, tumors [20], meningitis [17], glucose level abnormalities, non-cerebrovascular diseases such as epilepsy [21], dementia [22] and transient global amnesia [23]. Misdiagnosis of stroke has serious ramifications and therefore SMs must be excluded for proper identification of stroke subtype and treatment management. A recent prospective study of more than 300 patients with suspected stroke found 31% had SMs at the time of diagnosis [24]. Libman et al. were able to correctly identify and diagnose stroke in 81% cases finding 19% to be SMs [25].
TIAs, previously known as mini-strokes, are currently defined as ‘a brief episode of neurological dysfunction caused by focal brain or retinal ischemia with clinical symptoms typically lasting less than one hour and without evidence of acute infarction’ [2]. Strokes may sometimes be preceded by TIA and studies show that 35% of TIA patients have an increased risk of developing stroke within 5 years. [26]

**Clinical diagnosis**

One of the most critical elements in stroke diagnosis is the time of onset. In most cases, the arrival of the patient to the hospital is delayed due to the limited awareness of signs and symptoms of the disease. Rapid assessment of patients suspected with stroke is therefore highly crucial to accommodate the therapeutic treatment for any subtype of stroke by excluding SMs within a fairly short time window. Acute myocardial infarction is also one of the SMs noticed in some stroke patients. ECGs should be performed as a part of initial evaluation to rule out the possibility of a mimic in such cases. The major challenges associated with stroke diagnosis are the lack of imaging resources, lack of neurologic training in emergency clinicians, exclusion of SMs and misdiagnosis, and the limited time window for treatment.

**Stroke assessment scales**

Stroke scales are evaluation tools used to provide quick assessments of the severity of neurologic deficits including motor, sensory, visual and cognitive impairment and predict functional and global outcome arising from stroke conditions [27]. Implementation of a correct scale is extremely important and it must be simple, easily and expeditiously administered and sensitive to a patient’s condition.

Initial attempts were made to develop a unified scale but due to the complex nature and heterogeneity of stroke, separate scales were developed [28]. Based on the type of information collected concerning the patient, scales are divided into the following types: global outcome scales, which assess the outcome of the disability after stroke. The important ones include the modified Rankin scale (mRS), and the Glasgow outcome scale (GOS). Physical deficit scales, which provide information about the deficit with the resultant scores attached to severity, based on neurologic examination. Mathew stroke scale, Canadian neurologic scale, Scandinavian stroke scale, Orgogozo neurologic scale and the National Institutes of Health stroke scale (NIHSS) are some of the scales developed to measure neurologic impairment. So far, the NIHSS scale is the most widely used scale in stroke studies as it is a highly reproducible numeric scale and can be completed within a short span (5–8 min). It can be used both by neurologists and non-neurologists. Higher NIHSS scores indicate greater neurological deficits. Compared with other scales, this scale has proven intrarater and inter-rater reliability and has predictive
validity for stroke outcome [29–31]. Activities of daily living (ADL) scales, such as the Barthel index (BI), measures the functional outcome and recovery, useful in studies of rehabilitation. The diagnostic accuracy of most of these scales is approximately 80%, although their clinical sensitivity and specificity differ to some extent [32–35].

**Neuroimaging methods**

Neuroimaging has become an invaluable tool for the diagnosis of stroke and its subtypes. The advent of various imaging techniques has enabled clinicians to understand the cause of the disease and functional changes associated with the disease and guide future therapy to provide the utmost benefit to the patient by confirming the diagnosis. Stroke imaging serves two purposes: diagnose or confirm the occurrence of stroke by ruling out SMs and assess the location and amount of potentially salvageable brain tissue and irreversibly infarcted tissue. We briefly summarize some common neuroimaging techniques below.

The most commonly used imaging modality is CT because of its wide availability and its quick acquisition time compared with other imaging methods. CT is the ‘criterion standard’ for stroke evaluation and the primary diagnostic test for the detection of ICH and to rule out stroke mimics, such as brain tumors. Types of CT include noncontrast head CT (NCCT), CT angiogram of the head and neck (CTA) and perfusion CT (PCT) [36]. Of these neuroimaging modalities NCCT is the most widely used method to identify early signs of stroke and also to exclude or confirm hemorrhages. It’s availability and the speed of image acquisition makes it useful for initial evaluation of suspected stroke patients [37]. The utility of CT for stroke has been enhanced by the advent of newer generation CT scanners that permit additional imaging to be obtained with CTA and PCT [38]. However, the limitations of CT are exposure to ionizing radiation, side effects associated with the use of intravenous contrast agents and the inability of scanners to image the entire brain for perfusion imaging [39,40]. Recent advances in technology has enabled CT scanners to be equipped in ambulances and read the results via teleradiology [41].

MRI is another widely used neuroimaging method for the evaluation of stroke. This method is associated with high cost, limited availability and lengthy image acquisition times. But, MRI is considered to be extremely sensitive for detecting ischemic lesions. MRI can also detect or exclude ICH with an accuracy comparable to CT [42]. It has also been shown that the sensitivity of MRI is 83% and that of CT is 26% for the diagnosis of any stroke subtype [43]. Diffusion weighted imaging, perfusion weighted MRI, magnetic resonance angiography and fluid attenuated inversion recovery are some of the imaging techniques under the MRI umbrella [44].

**Therapeutics for stroke**

There is a very short therapeutic time window for stroke; window is defined here as the time between the onset of symptoms and treatment. For IS, the most common therapy is thrombolytic therapy, which restores blood flow to the damaged ischemic tissue by dissolution of the clot and reducing neuronal tissue damage [45]. Another approach is neuroprotection, which involves the use of drugs to protect the brain against neuronal dysfunction; their efficacy is limited due to associated side effects [13]. However, the combination of thrombolysis and neuroprotectants may offer advantages for the management of stroke [46].

**Recombinant tissue plasminogen activator & other acute stroke treatments**

Currently, the only FDA-approved drug for IS is recombinant tissue plasminogen activator (rt-PA). It works by attaching to the fibrin in the blood clots activating the production of plasmin, which causes clot dissolution. The findings of a National Institute of Neurological Disorders and Stroke (NINDS) trial have shown complete neurologic recovery in 31–50% of patients treated with rt-PA in 3 months [47]. However, rt-PA must be administered within a narrow time window of 3–4.5 h from the onset of stroke symptoms. Because a large number of patients arrive to the emergency room for neuroimaging diagnostics after this time window, administering proper treatment remains difficult. Therefore, studies have been conducted on the expansion of the effective therapeutic time window; results have shown that by increasing the time between symptom onset and treatment to 4–6 h makes the rt-PA treatment less beneficial [48–50]. However, rt-PA is beneficial in select patients up to 4.5 h post stroke [51]. Moreover, rt-PA is contraindicated in HS patients. Due to the imaging requirement and its lengthy analysis time, rt-PA only reaches 3–5%
of patients with IS in the USA [52]. Other perfusion enhancing approaches for IS are administration of aspirin and heparin [53]. However, possible benefits from aspirin and heparin could be obtained if administered within 48 and 3 h, respectively [54,55]. Additional treatments for acute IS are endovascular therapy in select patients, [56] ESCAPE [57] and EXTEND IA [58] along with aspirin.

**Role of blood–brain barrier in brain injury**
Understanding the physiology of the blood–brain barrier (BBB) is very crucial in developing effective diagnostics and therapies for brain diseases. The BBB is a dynamic barrier, which protects the CNS from unwanted and neurotoxic substances circulating in blood and allows for the transport and permeability of required nutrients. Any disruption or leakage of this barrier causes brain dysfunction and stimulates natural immune or inflammatory responses [59,60]. The BBB is comprised of endothelial cells forming the capillaries of the brain with tight junctions that restrict the movement of molecules into the brain and maintain the cell polarity, which refers to diversification of cell shapes through asymmetric cell divisions of stem cells that are crucial for proper functioning of differentiated cell types [61]. Other components of the BBB include pericytes, astrocytes and microglia, which support the permeability of this barrier and are involved in the immune responses of the CNS (see Figure 3) [62]. The BBB plays a major role in the immune system of the brain and any changes in the brain's microenvironment caused by stroke generates inflammatory responses due to activation of microglial cells and programed cell death (apoptosis) occurs [63,64].

Breakdown of the BBB and damage of endothelial cells following brain injury increases the permeability of the barrier and allows for the passage of potential biomarkers from the neurons and glial cells into peripheral blood [65]. There is also evidence of leukocyte infiltration into circulation in humans after stroke and it has been hypothesized that the accumulation of these leukocytes is the primary cause of tissue damage and prevents blood flow after restoration [66]. Specifically, neutrophil penetration into brain tissue causes significant damage due to the release of oxygen free radicals and proteolytic enzymes [67–70]. Also, certain biomarkers like matrix metallopeptidase-9 (MMP-9) play a biphasic role in stroke by disrupting the BBB during the initial phases of the stroke event and promote vascular growth during recovery phases [71].

---

**Figure 3. Cellular constituents of the blood–brain barrier.** The barrier formed by capillary endothelial cells, surrounded by basal lamina and astrocytic perivascular end feet. Astrocytes provide the cellular link to the neurons. Pericytes and microglial cells also form important components of a neurovascular unit. Reproduced with permission from [62] © Nature Publishing Group (2006).
Biomarkers are for stroke

Biomarkers are measurable indicators of normal or abnormal biological processes that may be used to screen for or diagnose a disease, monitor its activity, predict its course or assess response to treatment [72,73]. Cells, genes, hormones, enzymes or changes in biological function can be used as biomarkers [74].

Development of a proper diagnostic test for stroke through biomarker identification has remained a challenge for a variety of reasons including the complexity of the ischemic cascade and presence of the BBB. Identification of clinically relevant markers is a challenge as not all biomarkers are legitimate therapeutic targets. Using the appropriate biomarkers, especially blood-based biomarkers, can be extremely helpful for prehospital screening and to guide the intervention of thrombolytic treatment. Substantial efforts are ongoing to identify different biomarkers associated with stroke. With the existing diagnostic methods, time required to perform neuro-imaging and the limited availability of neuro-imaging equipment have prompted the need for a rapid diagnostic test that is capable of providing results within a few hours following stroke onset. A single biomarker, however, cannot be ideal for use as a standalone diagnostic test due to the complexity in etiology of stroke. In order to improve clinical outcome, development of a multi-biomarker panel, which can indicate brain damage by correlating to the volume of infarction and quick release into the blood/biological fluids upon stroke onset adds valuable and time-sensitive diagnostic information in the early evaluation of stroke, which can be further supplemented by CT or MRI results.

To date, no molecular diagnostic test exists for stroke and no biomarker has proven to be clinically sensitive and specific to diagnose either ischemic or hemorrhagic stroke. While no clinically useful biomarker exists for stroke, there are markers for coronary-related diseases, such as troponin or creatinine phosphokinase for the diagnosis of coronary syndrome, B-type/brain natriuretic peptide for congestive heart failure or d-dimer for pulmonary embolism, which have been used in point-of-care testing [75–77]. Finding ideal biomarkers for neuronal injury is complicated compared with heart diseases due to physiological differences, such as cellular heterogeneity in the brain (multiple cell types and cells with differentially localized elements, such as axons and cell bodies). Also, the BBB limits access of biomarkers in sufficient quantities to the vasculature unlike in heart diseases where there is direct access to the vasculature. Furthermore, complex biochemical pathways occurring in the ischemic cascade, their prolonged release following the stroke event and their inability to perfuse through the BBB [78] makes biomarker isolation difficult. Researchers also postulate that multimarker panels would increase clinical sensitivity for stroke diagnosis [79].

Investigations suggest that an ideal biomarker or a panel of biomarkers should be able to address the following diagnostic questions: does the patient have stroke; what is the mechanism of stroke- IS or HS; is there a need for initiation of thrombolytic treatment with the observed symptoms and is there a risk of recurrence? These questions can be addressed by finding an ideal biomarker that can be released into blood in a short amount of time in detectable quantities and the concentration levels should correlate with the volume of the infarct. Some practical considerations in the choice of these biomarkers include cost, factors that affect the measurement and the ease of obtaining the biomarkers. Ideal properties of biomarkers for clinical utility are summarized in Supplementary Table 1.

Protein biomarkers for stroke

This section highlights biomarkers relevant to IS only; very little research has been carried out on HS biomarkers due to fewer cases of HS. Biomarkers can be classified into several categories: imaging biomarkers, which measure changes in the nervous system through brain imaging; molecular biomarkers with biophysical properties and pharamacodynamic biomarkers indicative of certain pharmacological responses useful in drug development [80]. The present discussion is limited to molecular biomarkers (allow measurements in biological fluids, such as plasma, serum cerebrospinal fluid or blood). The onset of any brain disease triggers the release of biomarkers into cerebrospinal fluid and subsequently into the blood stream. Nevertheless, not all biomarkers released may be specific to stroke.

Protein biomarkers have been primarily studied due to better understanding of their relation with the pathophysiology of IS and their availability in most of the body fluids. Some of the biomarkers involved in ischemic injury include those associated with glial activation, inflammation, oxidative stress, thrombus formation, neuronal injury and endothelial dysfunction (see Supplementary Figure 1) [81,82].

Glial biomarkers

During the initial events in the ischemic cascade, glial cells are activated and inflammation is generated...
in response to ischemia. The most extensively studied markers specific to glial function are S100B, glial fibrillary acidic protein (GFAP) and myelin basic protein (MBP). In IS, serum levels of S100B are elevated within 12 h from symptom onset with continuous increase even after 3 days [83]. Even in HS, higher S100B concentrations have been observed [84,85]. It has been shown that S100B correlates with the volume of infarction, neurologic outcome and elevated levels of S100B in serum are believed to be due to BBB dysfunction [86,87]. Unfortunately, S100B is challenged as a stroke biomarker as its levels are found to increase in other neurological disorders (trauma, Alzheimer’s disease and schizophrenia), extracranial malignancies (schwannoma, melanoma and neuroblastoma) and also due its delayed release [88,89].

GFAP is a filament protein present in astrocytes and to a lesser extent in ependymal cells. It is a marker of glial cell lineage and has been reported to increase in stroke attacks with peak levels after 3 days [90,91]. A study by Foerch et al. showed that GFAP is a strong candidate for detection of HS due to its significantly increased levels observed in 81% of ICH patients compared with 5% of IS patients. Although increase in serum concentration of GFAP is correlated with volume of infarcted brain, its delayed release (6 h after symptom onset) limits its use for rt-PA administration [92,93].

MBP is a hydrophilic protein that plays an important role in the structure of myelin sheaths [94]. Increased levels of this protein are found in stroke and multiple sclerosis [95]. Detectable levels of MBP in IS subjects are observed after 1 week and levels decrease after 3 weeks [91]. The concentration of MBP were found to increase as indicated by NIHSS scores and large lesion volumes [96]. The limiting factor for the application of MBP as a diagnostic marker for stroke is its latency in response to a stroke event.

**Neuronal biomarkers**

During stroke, certain neuronal biomarkers are upregulated and released into blood as a result of CNS cellular responses. Increased levels of neuronal isoenzymes signal damage to the brain parenchyma [97]. Several neurobiochemical biomarkers that are evaluated in the case of neuronal injury have been identified and some of them are listed in Table 1.

Neuron-specific enolase (NSE) is a glycolytic enzyme specific for neurons. NSE levels are detect-

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description and function</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glial</td>
<td><strong>S100B</strong> Astrocyte marker; a calcium-binding protein involved in cell cycle progression &amp; differentiation [92,98]</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>GFAP</strong> Astrocyte marker; an intermediate filament protein role in cell structure, blood–brain barrier, communication [92]</td>
<td></td>
</tr>
<tr>
<td>Inflammatory</td>
<td><strong>IL-6</strong> Cytokine involved in acute-phase response and development of fever</td>
<td>[99]</td>
</tr>
<tr>
<td></td>
<td><strong>CRP</strong> An acute-phase reactant involved in atherothrombosis and ischemic injury</td>
<td>[100]</td>
</tr>
<tr>
<td></td>
<td><strong>MMP-9</strong> Proteolytic enzymes that degrade collagen-disrupting endothelium and plaque matrix [101,102]</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>VCAM-1</strong> Immunoglobulin involved in leukocyte – endothelial cell interaction</td>
<td>[103,104]</td>
</tr>
<tr>
<td></td>
<td><strong>TNF-α</strong> Cytokine involved in inflammation and acute-phase response</td>
<td>[99]</td>
</tr>
<tr>
<td></td>
<td><strong>ICAM-1</strong> Immunoglobulin involved in leukocyte – endothelial cell interaction</td>
<td>[104]</td>
</tr>
<tr>
<td></td>
<td><strong>ApoC-I</strong> Lipid metabolism</td>
<td>[105]</td>
</tr>
<tr>
<td></td>
<td><strong>ApoC-III</strong></td>
<td></td>
</tr>
<tr>
<td>Neuronal</td>
<td><strong>NSE</strong> Neuronal marker; a neuronal glycolytic enzyme [86,106]</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>FABP</strong> Cytosolic protein involved in long chain fatty acid transportation [106–107]</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>NDKA</strong> Glutamate excitotoxic response</td>
<td>[108]</td>
</tr>
<tr>
<td>Coagulation/</td>
<td><strong>vWF</strong> A glycoprotein involved in coagulation, platelet adhesion and factor VIII binding [109]</td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>D-dimer</strong> Marker of acute thrombosis; a breakdown product of fibrin blood clot</td>
<td>[110]</td>
</tr>
<tr>
<td>Other</td>
<td><strong>PARK7</strong> An RNA-binding protein; elevated in neurodegenerative disease</td>
<td>[111]</td>
</tr>
<tr>
<td></td>
<td><strong>NDKA</strong> Transfers phosphate between nucleosides</td>
<td>[111]</td>
</tr>
<tr>
<td></td>
<td><strong>BNGF</strong> A growth factor involved in neuronal growth and differentiation</td>
<td>[79]</td>
</tr>
</tbody>
</table>
able between 4 and 8 h of stroke onset with peak levels after 24 h [112]. Some of the limitations in using this biomarker include latency in release and less sensitive serum levels to brain tissue damage, although the sensitivity is high in CSF [113]. There is also no clear explanation on the variation of CSF and serum NSE levels, but serum NSE levels were significantly higher in the case of greater injury [114].

N-methyl-d-aspartate (NMDA) is an excitatory amino acid receptor and is a tetramer of two NR1 and two NR2 subunits [115]. A pilot study found increased serum levels of NMDA NR2 antibodies in patients with transient ischemic attack when compared with controls and even higher levels in IS subjects. These elevated levels correlated with NIHSS score and lesion volume [108]. Ongoing research is focusing on measuring NMDA receptor and its utility as a potential stroke biomarker.

Another neuronal biomarker, fatty acid–binding protein (FABP), is a cytosolic protein involved in transporting fatty acids. FABP is expressed in various tissues and rapidly released into circulation. In the CNS, heart-type FABP (H-FABP) is present in glia and brain-type FABP (B-FABP) is present in neurons at elevated levels within 2–3 h of stroke onset. Detection of this marker in a variety of other brain injury conditions does not make it stroke-specific [106–107,116].

Inflammatory biomarkers

One of the factors believed to cause neuronal death during an ischemic cascade is inflammation, which causes increased levels of inflammatory markers. These biomarkers are associated with increased infiltration of leukocytes. Biomarkers such as tumor necrosis factor-alpha (TNF-α) [117], matrix metalloproteinases (MMPs) [118], vascular cell adhesion molecule 1 (VCAM-1), C-reactive protein (CRP) [119], intercellular adhesion molecule 1 (ICAM-1) [120] and interleukin-6 (IL-6) [121] are some proteins involved in inflammation (Table 1). All of these biomarkers have shown only moderate clinical utility either due to low sensitivity and/or specificity [122].

Nucleic acid biomarkers for stroke

Alterations in gene expression in circulating leukocytes has been recently recognized as viable biomarkers for blood-based IS detection. Primary clinical gene expression profiling studies have taken advantage of and applied whole genome expression microarray technology for the discovery of new mRNA biomarkers. This approach permits unbiased selection of mRNA transcripts reflecting expression level of all known human genes and numerous noncoding sequences.

Moore et al. were first to publish gene expression profiling of peripheral blood mononucleated cells in patients with IS, using microarrays. After correcting for multiple comparisons and applying prediction analysis for microarrays, they identified a 22-gene profile classifying stroke with 78% sensitivity and 80% specificity in the validation cohort [123]. As few as four genes were found to provide accurate classification of IS. Two other clinical microarray studies identified panels of 18 and 9 gene mRNA transcripts in whole blood showing utility for IS detection [124,125]. The panel of 22 genes demonstrated greater than 85% accuracy when tested on a group of IS patients studied as early as 1–3 h after the onset of symptoms. In the study of Tang et al. changes identified within 1–3 h of symptoms, persisted up to 24 h.

The cellular sources of the expression changes are a key area of study as markers of greatest accuracy may be identified in individual leukocyte subsets. As the results of microarray studies were based on whole blood and PBMCs, cell subset specific results could not be assessed. However, it would be significant to compare the measured biomarker concentrations with the volume of the infarct from brain imaging to evaluate the clinical utility and predictive value of these blood biomarkers.

Stroke biomarker detection

Ideal biomarker detection strategies should be able to detect markers present in limited quantities in complex biological/clinical samples. Multiplexed immunoassays and quantitative mass spectrometry are two analytical platforms for the identification/detection of biomarkers. The type of analytical technique chosen depends on the question to be addressed and the availability of biological fluid for study, due to detection sensitivity associated with the analytical technique. After the discovery and verification of potential biomarkers, immunoassays may be developed and optimized to evaluate their clinical utility. Some of the most widely used immunoassays for biomarker detection will be discussed here.

Enzyme-linked immunosorbent assay

ELISA is routinely employed for the quantification and identification of protein, peptide, antibody and hormone biomarkers. Plate-based immunoassays
detect binding of an antigen to an antibody and uses an enzyme linked to a secondary antibody to generate a detectable product when the enzyme substrate is added (see Figure 4). For direct ELISA, the antigen is detected using an enzyme-conjugated primary antibody whereas unlabeled primary and conjugated secondary antibodies are used for indirect detection. The most sensitive and robust format of ELISA is the sandwich assay. In this format, the analyte is bound to capture and detection antibodies. The most commonly used enzyme labels are horseradish peroxidase (HRP) or alkaline phosphatase (AP). These enzymes produce a detectable signal that is directly correlated to the binding of antibody to an antigen.

A pilot study by Stejskal et al. used sandwich ELISA to evaluate the clinical utility of the visinin-like protein (VILIP-1), a CNS-abundant protein biomarker for brain injury and several neurogenerative diseases, which is released into circulation as a consequence of neuronal destruction. Serum and CSF levels of VILIP-1 were found to be elevated in IS patients approximately 3 h after stroke onset compared with healthy subjects. While the clinical sensitivity and specificity for stroke diagnosis were favorable, the assay and biomarker still requires further validation [128]. Another ELISA-based study using organ samples of mouse models enabled the measurement of VILIP-1 concentrations smaller than 100 ng/l and identified it as a potential candidate as a blood-borne biomarker of IS [129].

Further ELISA studies in a large cohort of European and American populations for stroke have shown increased concentrations of PARK7 (also called DJ-1) and nucleotide diphosphate kinase A (NDKA) in plasma samples after 3 h onset of a stroke event compared with a control population and remained increased up to 5 days after the initiation of the event. The authors hypothesized that the possible reason for overexpression of both PARK7 and VILIP-1 in plasma could be due to soluble protein produced by the injured neurons reaching the blood at the site of the lesion [111]. Zimmerman et al. used ELISA to detect heart-FABP (H-FABP) in the serum of stroke patients and healthy controls. Clinical sensitivity and specificity were reported to be 68 and 100%, respectively [130].

A new immunoassay, elongated oligonucleotide-linked immunosorbent assay (EOLISA), for protein biomarker detection has been reported [126]. This method utilized elongated DNA nucleotides (80mer), a complementary RNA oligonucleotide and RNase H for signal amplification (see Figure 4). This assay was used for the detection of H-FABP spiked into healthy human serum samples. When compared with conven-

![Figure 4. Immunoassays for detection of biomarkers. (A) Sandwich ELISA in which the target molecule is anchored to the substrate by capture antibodies and recognized by primary antibodies. The enzyme is linked to the immunocomplex through interactions between enzyme-decorated streptavidin and biotinylated secondary antibodies. Enzymatic biocatalysis generates a colored compound. (B) EOLISA, a sandwich format, which utilizes the biotinylated DNA strand and the fluorogenic RNA probes appended with a fluorophore and a quencher at each. At the signal amplification step, RNase H cleaves only the RNA part after forming RNA/DNA heteroduplexes, resulting in fluorescence recovery due to the release of fluorophore from the quencher. The iterative cycle of DNA/RNA duplexation and degradation of RNA by RNase H leads to the fluorescence signal amplification. EOLISA: Enzyme oligonucleotide-linked immunosorbent assay; P : Product; S : Substrate. (A) Reproduced with permission from [127] © Nature Publishing Group (2012). (B) Reproduced with permission from [126] © Elsevier (2013).]
tional ELISA, EOLISA showed tenfold higher detection sensitivity (0–1 ng ml$^{-1}$). Newer ELISA-based technologies such as Searchlight® and Fastquant® allow for operation with low sample volumes, decreased analysis time and simultaneous analysis of multiple samples unlike conventional ELISA-based methods [131]. Although ELISA is still considered a gold standard for biomarker detection, some challenges exist. Diagnostic assay development using ELISA is limited due to the fact that the limit-of-detection for ELISA is still in many cases not adequate to detect clinically relevant concentrations of biomarkers in samples at early disease onset [132] and the availability of high-quality antibodies are in some cases limited.

Miscellaneous detection approaches
Other approaches documented for the detection and validation of biomarkers for stroke include the use of a chemiluminescence immunoassay and micellar electrokinetic chromatography coupled to laser-induced fluorescence (MEKC-LIF). The most studied proteins for stroke, NSE and S100B, were detected using a chemiluminescence duplex immunoassay. It involved the detection of S100B, NSE with alkaline phosphatase (ALP) and horseradish peroxidase (HRP) labels, respectively; capture antibodies against markers were coupled to magnetic beads. Detection limits for S100B was found to be 0.005 ng/ml and NSE was 0.20 ng/ml [133].

MEKC-LIF with derivatization using 3-(2-furoyl) quinoline-2-carboxaldehyde (FQ) was optimized to separate and detect amines from plasma. MEKC-LIF was used to simultaneously detect five amine biomarkers, homocysteine (Hcy), excitatory amino acid (EAA) neurotransmitters principally including glutamic acid (Glu), polyamines such as putrescine (Put), spermine (Spm) and spermidine (Spd). From plasma samples of neuro-imaged IS patients, LODs of 0.2–2.1 nM (S/N = 3) were achieved, which is approximately 800-fold lower compared with the usual concentration levels of these analytes in human plasma under normal or pathophysiological conditions [134].

**Figure 5.** Design of the lab-on-a-chip detection area (left panel) consisting of magnetoresistive spinvalve sensors with each sensor made up of multiple alternating magnetic and nonmagnetic metal layers of 1 to 10 nm each. The resistance of the spinvalve sensor changes in response to an external magnetic field, which can be measured as an output voltage change. (A) Detection area consists of two rows of 12 sensors, (B&D) each sensor is made of nine spinvalves that are electrically connected in parallel. (C) The sensing area is covered with gold on which magneto-sandwich assay is built up. (E) Scheme showing the magneto-sandwich assay on the spinvalve sensing area coated with a self-assembling monolayer of alkane thiols onto which the primary antibody is immobilized. Secondary antibody binds to the proteins and forms a link with magnetic beads added in the final stage. Reproduced with permission from Parton E, Lagae L, Borghs G (IMEC) [140].

---

**Key terms**

**Quantitative qPCR:** Assay in which mRNAs are converted into cDNAs and then quantified using the polymerase chain reaction (PCR). This provides quantitative information as to gene activity and can be used for disease diagnostics in many cases.

**Lab-on-a-chip:** A device in which one or more laboratory functions are integrated onto a single wafer, such as a glass microscope slide. LOCs can process very small volumes of biological fluids in a fully automated fashion.
Detection of mRNA biomarkers
In clinical studies, quantitative polymerase chain reaction (qPCR) is the most accurate method. Hence it is used as a reference method for microarray studies and as large-scale validation methods for gene expression microarray studies. Available only recently, a microfluidic qPCR system (e.g., Biomark HD, Fluidigm) is available that can perform simultaneously up to 9216 reactions, which is a significant improvement over standard qPCR. Although reverse transcription and cDNA amplification is used, which are elements of all qPCR based methods, they can introduce inter-assay and interkit bias [135]. However, these qPCR gene expression results allow one to measure absolute gene expression with a theoretical limit-of-detection of a single copy of cDNA [136].

Lab-on-a-chip approaches & point-of-care testing
Point-of-care testing (POCT) refers to diagnostic testing performed outside the central lab not requiring the need for trained personnel to undertake the assay nor extensive levels of sophisticated instrumentation. The analytical targets for POCT include proteins, nucleic acids, metabolites and/or human cells from samples such as blood. For time sensitive conditions like stroke, developing a POCT diagnostic offers possibilities to accelerate stroke management by reducing times for transport, analyses or transmission of results to the physician [137]. Two types of POCT formats are bench-top analyzers and handheld portable devices. They require only basic instructions to use and provide easily interpretable results [138].

Two studies reported for stroke diagnosis using lab-on-a-chip (LOC) type devices included: a magnetoresistive spin valve detection platform (see Figure 5) for detecting the stroke diagnostic marker S100B. This protein is found in peripheral blood due to BBB disruption [139]. The concentration of S100B is 1–100 pg/ml in blood and this LOC platform was able to achieve a detection limit of 27 pg/ml [140].

Triage stroke panel and the associated hardware is an existing POCT platform that uses a panel of biomarkers associated with IS to provide diagnostic information. It is a fluorescence immunoassay used with the Triage Meters for the rapid, quantitative measurement of selected gene expression.
of B-type/brain natriuretic peptide, D-dimer, MMP-9 and S100B in whole blood or plasma specimens. The Triage meter plus (Biosite Incorporated, CA, USA) is a battery powered portable fluorometer that displays digital results in a turn-around-time of 20 min [141]. To eliminate the confusions in interpretation of clinical data due to multiple markers, Biosite® has developed an algorithm called the MultiMarker Index™ (MMX). The clinical performance of the Triage Stroke Panel assay has been assessed in a few studies [142–145] and it has shown utility and diagnostic value when combined with other diagnostic methods.

Our group has embarked upon building a fully automated LOC system for the analysis of peripheral blood and uses nucleic acid biomarkers (mRNAs) for stroke diagnosis. The processing pipeline consists of affinity selecting clinically relevant biological cells from peripheral blood, lysing the selected cells, solid-phase extraction to isolate mRNA molecules from the affinity selected biological cells and expression profiling mRNAs using a unique assay format (RT-LDR/spFRET; see below). The compelling aspect of this assay is that expression data can be secured in approximately 24 min and in a fully automated fashion, appropriate for POCT, especially for stroke diagnostics. The microarray assay requires approximately 11 h to secure the necessary data and is performed using specialized equipment and significant operator intervention that is not conducive to POCT. Below is a brief description of the devices made to carry out this assay.

A LOC device for the selection of biological cells from peripheral blood has been reported (Figure 6A) [146–148]. This LOC was fabricated from a plastic and consists of a series of channels containing monoclonal antibodies specific for the targeted cell (Figure 6A). The cell selection LOC (Figure 6B) was configured to simultaneously recover multiple cell types in blood [148] and subject the selected cells to mRNA expression analysis (Figure 6C). In this example, T-cells and neutrophils from blood were selected because these cells show expression differences resulting from IS or HS [148]. As can be seen in Figure 6C, CD66b positive neutrophils expressed higher levels of the S100A9 gene [149].

Expression profiling of mRNA using a single-molecule detection assay has been reported that can generate data in near real time to meet the requirements for stroke diagnostics. mRNAs were quantified by coupling reverse transcription (RT) and the ligase detection reaction (LDR) to single-pair fluorescence resonance energy transfer, spFRET (RT-LDR/spFRET), to obtain favorable limits-of-quantitation. In this assay, complementary DNA sequences (cDNA) are produced by reverse transcription of mRNA (see Figure 7A). A pair of oligonucleotide primers are designed to flank two adjacent sequences located within a unique region of the cDNA, which consists of a span of sequence that does not appear in any other cDNA species found in the sample. These primers also contain a 10-base stem sequence complementary to each other while not complementary to the target sequence. The end of each stem sequence contains either a donor or acceptor dye for fluorescence readout using spFRET that occurs when the stem sequences hybridize. Successful ligation of the LDR primers occurs only if the target sequence is complementary to both LDR primers resulting in the formation of a molecular beacon (MB). The MB adopts a stable stem-loop conformation following ligation forming the MB. The spFRET signal from individual MBs are read using a SMD instrument to obtain the copy number of the mRNA.

Near real-time results were obtained by carrying out the LDR thermal cycling and digital readout on a plastic LOC device. The LOC performed the LDR using a continuous flow format and then detected MBs online and in near real time (see Figures 7B & C). Expression profiling of genes related to stroke was evaluated and near real time reporting demonstrated the usefulness of the RT-LDR/spFRET assay [51].

Baird et al. discovered two genes, amphiphysin (AMPH) and IL1R2, that are expressed differentially among HS and IS patients; the discovery of these markers were affected through microarray studies [51]. Unfortunately, turnaround time of the assay and the complex nature of both RT-qPCR and cDNA microarrays can make these assay strategies difficult for monitoring mRNA transcript levels in a POCT for stroke diagnosis. As noted previously, there is only a 3–4.5 h effective window for treatment using rt-PA [152].

Using the RT-LDR/spFRET assay, expression analysis of AMPH transcripts were evaluated and rapid reporting capabilities of this assay was also demonstrated. The LDR was carried out using the microfluidic chip shown in Figure 7B and the assay time was approximately 2 min (includes LDR and fluorescence readout. Compared with RT-qPCR (R² = 0.98 over the same copy number range), the results were significantly improved using RT-LDR/spFRET with an R² value of 0.9995 over a copy number range of 0–10,000.

Conclusion & future perspective
Currently, stroke diagnosis is dependent on the patient undergoing a brain scan in order to differentiate between the major stroke subtypes (IS vs HS) and to rule out SMs. However, neuro-imaging methods
require the patient to be transported to the hospital and results in prolonged evaluation times for stroke patients. As such, less than 3% of those patients that are eligible to receive the FDA approved therapeutic, rt-PA, actually are given this drug.

Blood-based biomarkers could potentially be used for rapid stroke diagnosis and coupled with new measurement tools could serve as an attractive platform for managing many stroke-related diseases for POCT. In addition, the correct biomarker panel could also be envisioned for diagnosis of traumatic brain injury. Potential biomarkers include proteins and nucleic acids (mRNAs) isolated from circulating leukocytes. The challenge with any biomarker is the latency time associated with that biomarker appearing in peripheral blood. Unfortunately, in many cases potential markers do not appear in peripheral circulation well after the available therapeutic time window because the BBB must be compromised to allow these markers to appear in circulation. While leukocytes can be the carriers of relevant markers, alternatives may exist such as exosomes, which are constantly produced from biological cells and can potentially leak across the BBB providing earlier appearance of the appropriate biomarkers, such as mRNA.

New detection technologies must be generated as well because the need for rapid reporting is paramount irrespective of the type of marker used (i.e., protein vs nucleic acid). In particular, POCT technology that permits small blood volumes required for the analysis and rapid turn-around time measurement of multiple markers will enable the better management of stroke diseases. While current ELISA technologies are available for measuring serum protein levels, such as the TRIAGE system, there are issues with using protein biomarkers due to the relatively long latency time they display from disease onset to detectable levels appearing in the peripheral blood. This latency in many cases is mediated by the BBB, which must be compromised to generate sufficient levels of detectable protein biomarkers in peripheral blood.

Nucleic acid biomarkers for stroke diagnostics are attractive because their latency time can be shorter than protein biomarkers. However, their measurement is challenged by the extensive equipment and operator expertise required to generate the necessary analytical/clinical data. Our group is in the process of generating a technology that could permit results from multiple mRNA biomarkers within 20 min using less than 1 ml volume of blood and do so in a fully automated fashion appropriate for POCT.

To further understand the clinical utility of blood-based biomarkers for stroke diagnostics, further

---

**Figure 7. Single-Pair FRET analysis of mRNA transcripts.**

(A) RT-LDR/spFRET assay for the expression analysis of mRNA transcripts. Polymerase chain reaction is not used in this assay. Exquisite limits-of-quantitation with high analytical sensitivity are obtained by employing a linear amplification scheme and also due to the digital readout format (i.e., single-molecule counting). Following reverse transcription, flanking primer pairs targeting unique sequences in specific mRNA are used to identify specific mRNAs.

(B) Layout of the microchannels for the hot-embossed chip used to carry out a continuous flow LDR with on-line single-molecule detection.

(C) Photographic image of the actual chip.

discoveries and validation in research studies and clinical trials are needed. For example, discovering the cellular source of gene expression would allow identifying individual leukocyte subsets for stroke diagnostics and improve clinical sensitivity and specificity. With POCT systems equipped with highly sensitive biomarkers, more research studies and clinical trials could be conducted to identify and validate marker panels for differentiation and diagnosis of IS and HS. With the advent of new biomarker panels for stroke and the appropriate measurement tools of those markers, researchers could also identify lesion-impacted areas within the brain and the evolution of these lesions over time as well as their response to treatment.

Supplementary data
To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/full/10.4155/BIO.15.40

Financial & competing interests disclosure
The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Executive summary

Background
• Stroke is a disease resulting from deprivation of blood flow to the brain due to vascular occlusion or rupture.

Clinical diagnosis
• Current neuroimaging methods include computed tomography or MRI.

Therapeutics for stroke
• Recombinant tissue plasminogen activator (rt-PA) is the only FDA approved drug for ischemic stroke; less than 3% of patients receive it due to its narrow time window of effective use (3–4.5 h).
• Other neuroprotectants are still in a developmental phase of clinical trials and yet to be proved clinically effective.

Role of blood–brain barrier in brain injury
• A membrane is responsible for protecting the brain from neurotoxic substances; in the case of brain dysfunction and injury, they generate inflammatory responses allowing potential biomarkers from the neurons and glial cells to enter into the peripheral blood.
• Tissue damage in the brain will compromise the blood–brain barrier causing leukocyte infiltration and accumulation into peripheral blood.

Biomarkers for stroke
• Nucleic acid based biomarkers for stroke diagnostics are attractive because their latency time can be shorter than protein biomarkers.
• The challenge with existing protein biomarkers is the latency time associated with their appearance in detectable levels within peripheral blood.

Stroke biomarker detection
• Currently, ELISA technologies are available for measuring serum protein levels.
• Blood-based biomarkers coupled with new measurement tools could potentially be used for rapid stroke diagnosis, which can serve as an attractive platform for managing many stroke-related diseases.

Perspective
• To further understand clinical utility of blood-based biomarkers for stroke diagnostics, further discoveries and validation in research studies and clinical trials are needed.
• Point-of-care testing systems equipped with highly clinically sensitive biomarkers would allow to identify and validate biomarker panels for differentiation and diagnosis of ischemic stroke and hemorrhagic stroke with a rapid turn-around time.

References
Papers of special note have been highlighted as:
• of interest; •• of considerable interest


•• Reports the most recent and acceptable definitions of neurologic terms including Stroke transient ischemic attack based on the extensive studies done in the field.


specific enolase for prediction of regaining consciousness after stroke.


Foerch C, Montaner J, Sitzer M, Wunderlich M. Elevated serum S100B levels indicate a higher risk of haemorrhagic transformation after thrombolytic therapy in acute stroke. Akt. Neurol. 34(S 2), P575 (2007).


1057 Elucidated the first study gene expression profiling in peripheral blood in humans using microarray technology.
1059 Employed microarray technology to study the changes of gene expression during ischemic stroke using 18 gene panel.


