Review

Stroke Biomarkers:
Progress and Challenges for Diagnosis, Prognosis, Differentiation, and Treatment

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BACKGROUND: Stroke is a devastating condition encompassing a wide range of pathophysiological entities that include thrombosis, hemorrhage, and embolism. Current diagnosis of stroke relies on physician clinical examination and is further supplemented with various neuroimaging techniques. A single set or multiple sets of blood biomarkers that could be used in an acute setting to diagnosis stroke, differentiate between stroke types, or even predict an initial/reoccurring stroke would be extremely valuable.

CONTENT: We discuss the current classification, diagnosis, and treatment of stroke, focusing on use of novel biomarkers (either solitary markers or multiple markers within a panel) that have been studied in a variety of clinical settings.

SUMMARY: The current diagnosis of stroke remains hampered and delayed due to lack of a suitable mechanism for rapid (ideally point-of-care), accurate, and analytically sensitive biomarker-based testing. There is a clear need for further development and translational research in this area. Potential biomarkers identified need to be transitioned quickly into clinical validation testing for further evaluation in an acute stroke setting; to do so would impact and improve patient outcomes and quality of life.

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Stroke is the third leading cause of morbidity and mortality in the Western world, following ischemic heart disease and cancer. Globally there are more than 50 million stroke and transient ischemic attack (TIA)[3] survivors. More than 1 in 5 survivors will suffer a subsequent stroke in the following 5 years *(1)*, producing an immense burden on the economic and healthcare infrastructure. Recent estimates suggest the worldwide economic cost of stroke to be approximately $68.9 billion, including direct and indirect costs *(1)*. In the US alone there are an estimated 5–6 million stroke survivors, but with a cost of high disability. Between 15% and 30% of stroke survivors suffer permanent disability, and 20% of victims require institutional care within 3 months after the stroke event *(1, 2)*.

A variety of populations are at risk for stroke, and the disease should no longer be considered confined to the elderly, since a third of stroke victims are under the age of 65. Blacks have twice the risk of stroke compared with whites, and women are at a greater risk for stroke than men. In 2005, women accounted for 60.6% of stroke deaths in the US; the increased incidence is primarily due to an increased lifespan *(1)*. However, anyone is at risk for stroke if they have concurrent or previous inflammatory and vascular risk factors, including myocardial infarction, coagulopathies, peripheral vascular disease, hypertension, atrial fibrillation, or diabetes mellitus.

Classification of Stroke

Stroke terminology encompasses a vast composition of pathophysiological entities that include thrombosis, embolism, and hemorrhage. Broadly, stroke is classified as ischemic or hemorrhagic types, with ischemic stroke accounting for approximately 85% of the total number *(1, 3)*. Ischemic stroke is primarily caused by either intracranial thrombosis or extracranial embo-

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[3] Nonstandard abbreviations: TIA, transient ischemic attack; ICH, intracerebral hemorrhage; SAH, subarachnoid hemorrhage; CSF, cerebrospinal fluid; NIHSS, National Institutes of Health Stroke Scale; CT, computed tomography; MRI, magnetic resonance imaging; NCCT, noncontrast CT; DWI, diffusion-weighted imaging; rtPA, recombinant tissue plasminogen activator; FDA, US Food and Drug Administration; Lp-PLA2, lipoprotein-associated phospholipase A2; ARIC, Atherosclerosis Risk in Communities; CRP, C-reactive protein; WHI, Women's Health Initiative; ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; LC-MS/MS, liquid chromatography–tandem mass spectrometry; OR, odds ratio; RR, relative risk; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; NMDA, N-methyl-D-aspartic acid; NR2Ab, NMDA receptor antibody; GFAP, glial fibrillary acidic protein; NSE, neuron-specific enolase; APC-PCI, activated protein C–protein C inhibitor complex; NDKA, nucleoside diphosphate kinase A; BNP, brain natriuretic peptide.

Table 1. Clinical conditions that may mimic stroke [Adams et al. (7)].

Seizure
Hypoglycemia
Drug overdose
Hyponatremia
Migraine
Brain tumor
Subdural hematoma

lism. Intracranial thrombosis is largely due to atherosclerosis, whereas extracranial embolisms commonly arise from the extracranial arteries or from the myocardium due to concurrent myocardial infarction, mitral stenosis, endocarditis, atrial fibrillation, dilated cardiomyopathy, or congestive heart failure. Hemorrhagic stroke can be classified as either intracerebral hemorrhage (ICH) or subarachnoid hemorrhage (SAH). ICH originates from weakened cerebral vessels, which rupture and form a localized hematoma within the parenchymal cerebral space. In SAH the hemorrhage occurs outside of the brain and is released into the cerebral spinal fluid (CSF). The common causes for both ICH and SAH are comparable and include hypertension, trauma, drug use, or vascular malformations.

A TIA, known as a "ministroke," elicits focal neurological deficits similar to an ischemic stroke but has historically been defined as lasting <24 h (4). However, it is well known that the majority of TIAs resolve within 1 h (5), and 90% completely terminate after 4 h (6). Therefore, the American Heart Association has recommended that a TIA be defined based on evidence of a transient episode of central nervous system tissue ischemia without infarction, and not simply on an arbitrary time point (4). This definition is controversial but places high value on an accurate and rapid diagnosis of TIA, since TIAs are strong predictors of short-term risk for a full ischemic stroke, cardiovascular events, and death. This risk can be mitigated through early administration of thrombolytic therapies. Diagnosis of TIAs, as well as detailed discussion surrounding risk stratification, will not be discussed in this review but may be found elsewhere (4, 7).

Finally, the term "stroke mimics" is used to encompass a variety of abnormalities that imitate the signs and symptoms of stroke (Table 1). The most common stroke mimics include hypoglycemia and seizure, 2 clinical conditions frequently encountered in an emergency setting. Stroke mimics can confound the rapid diagnosis and treatment necessary for an optimal outcome in stroke victims.
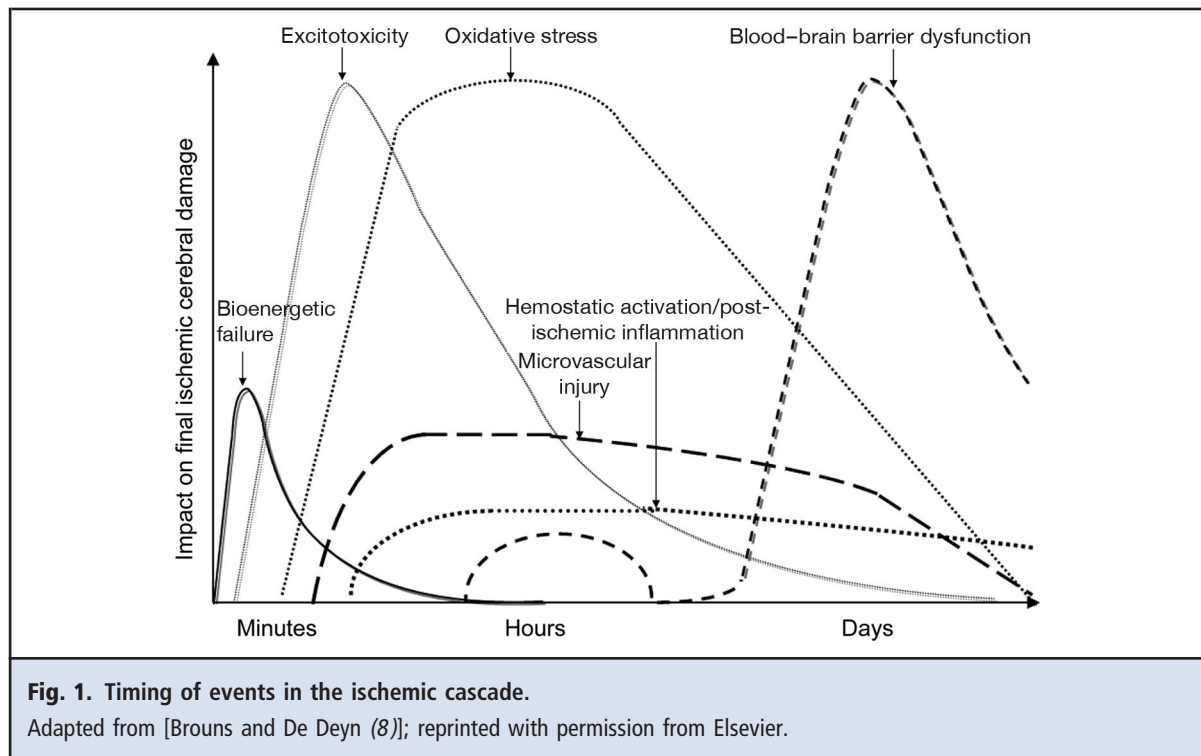
Stroke Pathophysiology

The complexities embedded within the pathophysiological pathways of stroke are numerous, although there are many similarities and differences between ischemic and hemorrhagic stroke. Ischemic stroke initiates a generalized series of events occurring at the onset of cerebral ischemia, referred to as the ischemic cascade (Fig. 1). The overall and exact timing of each event is heterogeneous and depends on many variables such as the infarct size, onset and duration of ischemia, and effectiveness of reperfusion. The ischemic events initiate with gradual or sudden cerebral hypoperfusion and include cellular bioenergetic failure, excitotoxicity, oxidative stress, blood–brain barrier dysfunction, microvascular injury, hemostatic activation, inflammation, and eventual necrosis of neuronal, glial, and endothelial cells (8). Disruption of the blood–brain barrier in ischemic stroke appears to be a biphasic event and relies on the aggressiveness and response to reperfusion. Within the first 24 h of an ischemic stroke, there is increased permeability of the blood–brain barrier, and further damage occurs 48–72 h after infarction.

The major cause of intracerebral hemorrhagic stroke remains attributed to chronic hypertension causing weakened blood vessels, and despite some controversy there have been no substantial increases in the prevalence of ICH in spite of an increase in anticoagulant usage. The onset of symptoms in ICH may also be rapid or gradual, and clinical outcomes depend highly on the volume and expansion of the hematoma. Within the first few hours after ICH, varying degrees of edema occur and result in clot retraction and release of osmotically active proteins into the surrounding tissues (9, 10). After 2–3 days, activation of the coagulation cascade ensues and is coupled with synthesis of thrombin and a systemic inflammatory response. Finally, there is neuronal hemoglobin toxicity and erythrocyte lysis, which occur several days after the initial ICH event. Challenges toward discovery of a stroke biomarker for hemorrhagic stroke involve the delayed disruption of the blood–brain barrier, which remains intact for large molecules for several hours posthemorrhage. Only after a sizeable increase in hematoma volume (on average 8–12 h later) will permeability of the blood–brain barrier become substantial enough to measure brain-specific proteins.

The intricacy and diversity of brain tissue types, combined with a lack of knowledge regarding the complete cerebral physiology, contribute to the current paucity of stroke-specific biomarkers.

Fig. 1. Timing of events in the ischemic cascade.
Adapted from [Brouns and De Deyn (8)]; reprinted with permission from Elsevier.

Clinical Diagnosis of Stroke

Clinical diagnosis, differentiation, and management of stroke are predicated on obtaining an accurate medical history and a thorough physical assessment of the patient. Acute onset of speech disturbance and focal weakness are hallmark symptoms of both ischemic and hemorrhagic stroke. The diagnostic accuracy of stroke has been shown to have a sensitivity of 92% in primary care physicians routinely exposed to suspected stroke victims (11), but is less reliable in physicians with less experience or confidence (12). Rapid assessment of acute stroke victims is critical for determining eligibility for thrombolytic therapy, as the window of opportunity for therapeutic effectiveness of stroke is very narrow, only a few hours, compared with myocardial infarction. To assist with diagnostic consistency among physicians, the 42-point National Institutes of Health Stroke Scale (NIHSS) scale was developed and designed to be completed within 5 to 8 min (7, 13). The NIHSS quantifies neurological deficits in stroke patients and has prognostic value for predicting the progression of complications. The NIHSS has not yet demonstrated an independent improvement in patient outcomes.

Neuroimaging remains the only tool available for differentiating between ischemic stroke and ICH, as the symptoms of the 2 conditions show substantial overlap. Individuals with SAH often present with no focal signs or symptoms because the hemorrhage is extracerebral; however, ICH patients commonly refer to having an extreme, intense headache of sudden onset. Diagnostic criteria for stroke do not use specific blood biomarkers, instead relying solely on clinical assessment and interpretation of radiological findings (Table 2).

Imaging Techniques

A variety of imaging techniques are available, and the advent of new research findings have led to the potential for earlier opportunities for therapeutic intervention in stroke patients. The primary goal for the initial neurological imaging studies is to differentiate between hemorrhagic and ischemic stroke or exclude stroke mimics in a timely manner. Many important features gained from brain imaging ultimately help guide treatment decisions or options, which include detecting early infarction and determining the location and degree of infarct and vascular distribution of the lesions responsible for the stroke. Computed tomography (CT) and magnetic resonance imaging (MRI) are routinely used in the initial acute assessment of stroke victims.

The main advantages of CT are its widespread availability and the timely manner in which the scan

Table 2. Diagnostic tests used in the acute evaluation of stroke [Adams et al. (7)].	
All patients	**Selected patients**
Neuroimaging (NCCT or MRI)	Chest X-ray
Electrocardiogram	Liver function tests
Cardiac markers (troponin)	Arterial blood gas
Complete blood count	CSF analysis
Platelets	Lipid profile
Electrolytes	Toxicology screen
Glucose	β-hCG
Coagulation tests (PT/INR, aPTT)[a]	Blood alcohol
Renal function tests	Electroencephalogram
Oxygen saturation	

[a] PT, prothrombin time; INR, international normalized ratio; aPTT, activated partial thromboplastin time; β-hCG, beta-human chorionic gonadotropin.

can be performed. In the hyperacute phase, noncontrast CT (NCCT) is widely accepted as the standard neuroimaging technique, and NCCT is typically conducted in all suspected stroke patients after medical stabilization to detect cerebral lesions or acute hemorrhage (7). Newer multimodal techniques (NCCT combined with perfusion imaging and angiography) are used to counteract some of the downfalls of NCCT, which is not sufficiently sensitive to accurately diagnose ischemic stroke owing to the inability to visualize the full vascular occlusion and degree of collateral circulation and insensitivity toward early ischemia (14).

New imaging modalities have challenged the use of multimodal CT in a routine manner. In the setting of acute stroke, MRI diffusion-weighted imaging (DWI) techniques have the ability to differentiate between various stroke subgroup populations and have consistently demonstrated superior sensitivity in the first hours after stroke compared with NCCT (95%–100% vs 42%–75%, respectively) (15, 16). MRI has been shown to detect about one-half of all cases of TIA (17). MRI-DWI techniques can also yield additional information in stroke patients who delay seeking treatment. Schulz et al. (18) conducted a prospective observational study of 300 patients with suspected stroke or TIA and a median delay of 17 days from symptom onset. In this cohort, use of MRI-DWI yielded a clarification of diagnosis or vascular territory that altered the management in 42 (14%) of patients. MRI-DWI also assisted in the evaluation of acute ischemic stroke, and the presence of multiple DWI lesions on the baseline MRI scan was associated with an increased risk of early lesion reoccurrence (19–21). The presence of multiple lesions on MRI-DWI, regardless of lesion age, was an independent predictor of future ischemic events (22).

Although there is improved resolution with MRI-based techniques, some of the obstacles of routine implementation include the limited availability and high cost of the scanners. Current recommendations suggest the use of MRI in patients eligible for thrombolytic therapy only if studies can be completed in the same amount of time as NCCT studies (7). Reduction of the MRI scan time is an area of active research, and protocols are being developed that reduce the acquisition time from 15–20 min down to ≤5 min (23, 24).

Despite advances in the field of neurological imaging, there are inherent limitations to CT and MRI. Of logistical significance, imaging currently requires substantial time for the procedure and clinical interpretation. In addition, analyses of radiological images are prone to intraindividual variation (25–27). In contrast to biomarkers, the equipment required in obtaining CT or MRI scans will likely never allow availability in the field, where this information would be of substantial value.

Therapeutic Intervention in Stroke

Effective thrombolytic therapy must be initiated rapidly to salvage as much cerebral tissue as possible. Intravenous recombinant tissue plasminogen activator (rtPA) has revolutionized therapy in acute stroke since approval by the US Food and Drug Administration (FDA) in 1996, and has been used consistently for thrombolysis in acute stroke. The window of opportunity for thrombolytics is 4.5 h from the onset of symptoms (28), and therefore time to diagnosis is critical. In addition, there is an extensive list of contraindications for administration of rtPA, most notably in patients who have had stroke/trauma or myocardial infarction in the prior 3 months, seizures, hypertension, hypoglycemia, symptoms of SAH or evidence of ICH on imaging, or low platelet count.

In the US, about 22% of ischemic stroke patients present to the emergency department within 3 h, but only 8% of those individuals meet all the inclusion criteria for rtPA therapy (29–31). It is recommended that treatment with rtPA not be delayed before imaging studies in eligible patients, as the benefits have been shown to outweigh the risks (7). If stroke could be diagnosed earlier, or with greater certainty, the therapeutic options could be greatly improved. Furthermore, therapeutic intervention remains an area where there is great importance in absolute differentiation between ischemic stroke and ICH, because misclassification of ICH as ischemic stroke would be lethal if thrombolytics were mistakenly administered.

The Need for Stroke Biomarkers

Cerebral infarction biomarkers have the potential to alter and expedite the differential diagnosis and prediction of stroke, particularly in challenging cases where the neuroimaging findings appear normal or equivocal. Difficulties in biomarker discovery revolve around the slow release of glial and neuronal proteins across the blood–brain barrier after stroke or traumatic injury. In addition, cerebral ischemia markers can lack diagnostic specificity and are increased in a variety of the stroke mimic situations. Ideal stroke biomarkers should exhibit characteristics that include diagnostic specificity and sensitivity to infarcts, differentiation between hemorrhagic vs ischemic stroke, early and stable release shortly after infarction, predictable clearance, potential for risk assessment and guidance of therapies, and the ability to be quantitatively and rapidly measured by cost-effective methodologies.

Improvement in patient outcomes in the setting of acute stroke necessitates a rapid and accurate diagnosis of stroke, and clearly stroke biomarkers have the potential to assist in both prediction and diagnosis of stroke. We next examine some of the biomarkers predicated on positive and negative study results from experimental research and clinical trials.

Biomarkers for Risk Prediction and Diagnosis of Stroke

LIPOPROTEIN-ASSOCIATED PHOSPHOLIPASE A2

Lipoprotein-associated phospholipase A2 (Lp-PLA2) is a 50-kDa calcium-independent serine lipase that hydrolyzes oxidized phospholipids to release proinflammatory lysophosphatidylcholine and oxidized fatty acids *(32)*. Lp-PLA2 binds and circulates bound to LDL and is particularly drawn to small, dense LDL particles. Depending on the extent of Lp-PLA2 glycosylation, it may also bind small, dense HDL particles thought to contribute to an antiatherogenic mechanism. Lp-PLA2 is produced and expressed in macrophage-rich atherosclerotic lesions and is markedly upregulated in advanced coronary lesions. The FDA has approved use of Lp-PLA2 for long-term prognostic risk for coronary heart disease and stroke; it confers about a 2-fold increase in stroke occurrence *(33)* and of recurrent stroke with an adjusted hazard ratio (HR) of 2.54 (95% CI 1.01–6.39) *(34)*. Lp-PLA2 has emerged as an independent inflammatory marker of cardiovascular risk and predictor of ischemic stroke events based on the outcomes of several large clinical trials.

A positive association between plasma Lp-PLA2 concentration and the risk for ischemic stroke was demonstrated in the Rotterdam trial, a retrospective-prospective study of nearly 8000 men and women over the age of 55. In this cohort, ischemic stroke occurred in 110 individuals during a median follow-up time of 6.4 years, and the age-/sex-adjusted hazard ratio was 2.0 between the first and fourth quartiles for Lp-PLA2 activity *(35)*. Alternate lipid parameters (total cholesterol and non-HDL cholesterol) were identical in stroke patients vs controls. Of similar design, the Atherosclerosis Risk in Communities (ARIC) study identified 194 cases of ischemic stroke during 6-year follow-up, with significant mean baseline plasma Lp-PLA2 differences between stroke and control groups (443 and 374 μg/L, respectively, $P < 0.001$) *(36)*. Lp-PLA2 and C-reactive protein (CRP) concentrations were complementary in the identification of stroke risk in this study; individuals with Lp-PLA2 in the third tertile ($\geq$422 μg/L) and CRP >3 mg/L had more than 11-fold higher risk for ischemic stroke than those with Lp-PLA2 in the first tertile (<310 μg/L) and CRP <1 mg/L *(36)*. Regardless of LDL cholesterol concentration, Lp-PLA2 independently predicted stroke (HR 2.08; 95% CI 1.20–3.62), suggesting that although Lp-PLA2 is carried by LDL particles, its presence may convey a different risk than LDL alone.

The Women's Health Initiative (WHI) trial evaluated the prospective risk of ischemic stroke in low-risk postmenopausal women in 40 different clinical centers across the US and yielded results that were less enthusiastic *(37)*. In this population, the risk of incident stroke was indeed significantly greater in study participants with an increased Lp-PLA2 compared with controls (929 strokes vs 935 matched controls, $P < 0.003$) *(38)*. However, the relative risk per standard deviation increase in the risk of ischemic stroke was 1.07 (95% CI 1.01–1.14), with the significance driven by a greater occurrence of large- but not small-vessel stroke. Also, in contrast with other studies, there was no association between the risk of stroke and increased concentrations of CRP.

Lp-PLA2 assays measure either mass or activity, and there is little consistency regarding which type of assay is used from 1 study to the next. A single company currently markets the FDA-approved mass assay for Lp-PLA2 (PLAC assay; diaDexus), and all activity assays are for research use only. Lp-PLA2 mass and activity are incompletely correlated, likely because of variation in the substrate used in activity assays. The results in activity assays depend on the substrate, and it is unclear how other plasma phospholipases interact with the substrate used; this source of potential analytical variability has not been well characterized. It is noteworthy that the Lp-PLA2 mass assay may have its own analytical challenges: studies using mass measurements have drawn variable conclusions, perhaps related to the questionable instability of current third-generation assays *(39)*. If strategies for lowering Lp-PLA2 using oral

inhibitors such as darapladib prove to have benefit in stroke and cardiovascular risk reduction, it will be critical that standardization of mass and activity assays occurs. Although full analytical characterization is needed to bring Lp-PLA2 into optimal clinical use, available evidence indicates that it is a potentially important marker for prediction of stroke risk.

ASYMMETRIC DIMETHYLARGININE

Methylarginines are synthesized by posttranslational methylation of L-arginine and are released as free dimethylarginines after proteolysis. Asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are detectable in blood, urine, and CSF. Whereas SDMA is inactive, ADMA is a potent inhibitor of nitric oxide synthase, which mediates widespread endothelial dysfunction. Therefore, increased plasma ADMA is hypothesized to be a marker for stroke risk prediction and has been associated with other traditional cardiovascular risk factors including hypertension (40–42), diabetes (43, 44), hyperhomocysteinemia (45–47), left ventricular hypertrophy (43, 48), and hypercholesterolemia (49–51). ADMA is accurately quantified by ELISA or HPLC/liquid chromatography–tandem mass spectrometry (LC-MS/MS), methods that achieve the necessary precision and separation of ADMA from other structural isomers.

In several clinical studies, plasma ADMA has been shown to correlate with stroke risk. Yoo and Lee (52) recruited 52 stroke patients and 36 healthy controls and demonstrated significant differences in ADMA concentrations between those with recurrent stroke (mean 2.28 μmol/L), initial stroke (mean 1.46 μmol/L), and controls (mean 0.93 μmol/L) ($P = 0.0001$). Increases above the 90th percentile of the control group ($\geq$1.43 μmol/L) increased the overall stroke risk in the elderly population studied [odds ratio (OR) 6.05, 95% CI 2.77–13.3, $P = 0.02$]. The Population Study of Women in Gothenburg evaluated ADMA in 880 women and demonstrated that small increases (0.15 μmol/L) in ADMA over a period of 24 years were associated with a 30% increase in stroke and myocardial infarction (53), and ADMA concentrations in the top quintile ($\geq$0.71 μmol/L) conferred the greatest relative risk (RR) (1.75; 95% CI 1.18–2.59). Also, the Framingham Offspring Study evaluated plasma ADMA concentrations from 2013 individuals for whom simultaneous neuroimaging studies were available (54). ADMA was independently associated (OR between quartile 1 and quartiles 2–4: 1.43, 95% CI 1.00–2.04) with an increased prevalence of MRI abnormalities and lesions in the absence of clinical symptoms, which is a significant risk factor for preemptive stroke.

Overall, ADMA appears to be a novel biomarker linked to overall cardiovascular mortality, endothelial dysfunction, and risk of stroke, and further studies are clearly warranted to validate its clinical utility.

MATRIX METALLOPROTEIN 9

Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent endopeptidases responsible for turnover and degradation of extracellular matrix proteins. Regulation of MMP activity is important in tissue remodeling, inflammation, angiogenesis, and tumor cell metastasis (55, 56). Secreted as zymogens (pro-MMPs), MMPs are activated by a variety of proteinases, and their activity is highly regulated by interaction with tissue inhibitors of metalloproteinase (TIMPs) and by α2-macroglobulin. Cerebral tissue expression of MMP-9 is normally minimal to undetectable, but increases in MMP-9 were discovered in the ischemic brain more than a decade ago (57, 58). Upregulation of MMP-9 occurs within the brain in response to injury and is hypothesized to play a central pathological role in stroke through degradation of extracellular matrix proteins that are essential to maintain homeostasis. After the onset of stroke, uncontrolled MMP expression and activity mediates proteolysis and leads to blood–brain barrier leakage and cell death (59–62).

The release pattern of MMP-9 is not well characterized, but increases are seen in both ischemic and hemorrhagic stroke patients upon presentation to the emergency department compared with healthy individuals, which suggests a relatively short time period (hours) from release to detection (63, 64). Acute MMP-9 concentrations have also been related to infarct size, poor neurological outcome, and hemorrhagic transformation complications (63, 65, 66). MMP-9 concentrations assessed at hospital admission have been identified as a predictor of infarct volume as measured with diffusion-weighted MRI (67), and the biomarker is further correlated with stroke lesion growth, even with the effective application of thrombolytic therapy (68). In addition, an initial study suggested that compared with other therapies such as hypothermia, MMP-9 concentrations were increased in patients that received rtPA, suggesting a possible "washout" phenomenon (69). A more recent study confirmed that blood MMP-9 concentrations in stroke patients treated with rtPA were significantly higher than those in untreated patients (70). Consistent with the hypothesis of deleterious MMP actions during ischemic stroke, hyperacute MMP-9 blood concentrations emerged as a predictor of further hemorrhagic complications after rtPA administration (67). Technically, all assays for MMP-9 are enzyme immunoassays, which are not standardized; therefore cutoffs and assay characteristics cannot be superimposed from 1 study to the next.

MMP-9 likely mediates a dual role in stroke pathogenesis, which includes disruption of the blood–brain barrier, neuronal cell death, and hemorrhage after stroke, and a healing role during brain regeneration and neurovascular remodeling in the later tissue repair phase. Experimental and clinical data with MMPs are promising, as the majority of studies demonstrate a clear correlation of MMPs with MRI and neurological outcomes in stroke.

S100 BETA

S100 β (S100B) is a low molecular weight glial protein (approximately 10 kDa) that belongs to a multigenic family of calcium-mediated proteins (S100 proteins), so named for their solubility in 100% ammonium sulfate (71). Various combinations of subunits (α and β) make up the S100 protein family, which diverge into the hetero- and homodimer forms of α–α, α–β, and β–β subunits. S100B is comprised of the β–β and α–β forms, is highly specific to nervous tissue, and is found in abundance in the cerebral astroglial compartment, peripheral Schwann cells, and extraneuronally in melanocytes, adipocytes, and chondrocytes (72). S100B is hypothesized to be a marker of generalized blood–brain barrier dysfunction rather than specific glial damage owing to its broad localization in various cell types (73). S100B is released into CSF upon structural damage to the neuronal cells, but the underlying mechanism of passage through the blood–brain barrier has not been clearly elucidated. The concentration of S100B is 40-fold higher in CSF than in serum. The biomarker is not affected by hemolysis and has exceptional stability (74), giving it appeal for use as a clinical biomarker.

Several studies have demonstrated that serum S100B concentrations are increased significantly following stroke (75–82), with secretion of S100B increasing up to 48 h after symptom onset and the peak concentration occurring within the first 24 h after cerebral infarction. Elting et al. (78) reported that patients who had a TIA or normal brain CT at presentation had significantly lower S100B concentrations with minimal variation over time, in comparison with individuals who had major neurological deficits and abnormal brain imaging showing large-artery cortical infarcts. The obvious pitfall for widespread use of S100B in acute situations includes its apparent prolonged and delayed release into the blood. At this time, the lack of diagnostic sensitivity of serum S100B precludes its diagnostic use in acute stroke situations.

Significant correlations between S100B concentrations in blood and the size of infarction area were demonstrated in a variety of clinical or experimental studies on focal ischemia. Jonsson et al. (83) demonstrated that lesion size strongly correlated with S100B concen- trations 48 h after cardiac surgery in cases with focal ischemia as a secondary complication. A few studies have reported a direct correlation of stroke severity to S100B concentrations. Jauch et al. (80) found higher S100B concentrations to be statistically associated with greater baseline NIHSS values (r^2 = 0.263, $P < 0.0001$). Hill et al. (84) also demonstrated peak concentrations of S100 to be significantly correlated with admission NIHSS scores ($P < 0.05$).

Increased S100B in blood is not specific for cerebral infarction, as increases occur in other neuropathologies including traumatic brain injury and extracranial malignancies, thus yielding the potential to skew interpretation of results (72, 85, 86). Overall, the clinical performance of S100B is lackluster in diagnosis and differentiation between acute ischemic, hemorrhagic, or stroke mimics. Thus, it does not appear that S100B will be a useful biomarker in the clinical context of stroke, and measurements may be reserved for evaluation of brain injury and trauma.

NMDA RECEPTOR PEPTIDES AND ANTIBODIES

N-methyl-D-aspartic acid (NMDA) receptors bind the glutamate neurotransmitter and are heterogeneous on neuronal cells throughout the brain. NMDA receptors typically contain 4 subunits, 2 NR1 and 2 NR2 subunits, and fragmentation of NR2 into NR2A and NR2B peptides is thought to occur with cerebral ischemia or neurotoxicity. Generation of NMDA receptor antibodies (NR2Abs) is mediated by the immune system following ischemic events, and either these autoantibodies or the NR2 peptide fragments themselves can be quantified in CSF and blood.

A few clinical studies have examined the role of NR2Ab and NR2 peptides as markers of stroke. Using an ELISA, Dambinova et al. (87) measured autoantibodies to NR2A/2B in 105 stroke or TIA patients and 255 controls. NR2Abs were detected in significantly higher quantities in the ischemic stroke and TIA patients vs controls ($P < 0.0001$), but the antibody concentrations could not be used to differentiate ischemic stroke from TIA. NR2Abs were not increased in ICH patients or the control group, suggesting that a negative NR2Ab result does not rule out ICH. In this instance, imaging would remain the standard of care. At a cutpoint of ≥ 2.0 μg/L, there was a high sensitivity (97%) and specificity (98%) for the diagnosis of ischemic stroke or TIA within 3 h of symptom onset. The positive predictive value was 86% for ischemic stroke and 91% for TIA, and the negative predictive value was 98% for both ischemic stroke and TIA. Increased autoantibody concentrations were observed in hypertensive individuals and those with a history of stroke or atherosclerosis. Because the latter factors predict stroke risk, however, it was unclear whether the increased au-

toantibody concentrations reflected current stroke episodes or were potential predictors of future cerebrovascular events. In a prospective, multicenter clinical trial, NR2Ab concentrations were evaluated for their ability to predict adverse neurological complications in 557 patients undergoing coronary surgery (88). Only 25 patients had an NR2Ab preoperative concentration ≥2.0 μg/L, but 24 of 25 proceeded to develop neurological complications within 48 h postoperatively (RR 17.9, 95% CI 11.6–27.6). Therefore, NR2Ab may be useful for prediction of neurological events in high-risk individuals.

In vivo production of autoantibodies inherently requires some amount of time and potentially limits the utility of serum NR2Ab measurement immediately after the onset of stroke symptoms. For this reason, NR2 peptide fragments may prove to be a more suitable measurement target, as the fragments are likely formed immediately after the ischemic event. NMDA receptor assays yield promise as upcoming stroke biomarkers, and replication of study results in larger multisite trials is necessary to eliminate potential measurement bias.

GLIAL FIBRILLARY ACIDIC PROTEIN

Glial fibrillary acidic protein (GFAP) is a monomeric filament protein specific to the brain astrocytes (89). Although the exact role of GFAP is unknown, it is involved with various neuronal cellular processes and is partially responsible for neurological functions within the blood–brain barrier. Initial clinical studies with GFAP demonstrated increased serum concentrations in ischemic stroke patients vs controls, with peak concentrations occurring 2–4 days after symptom onset (90–92). The prolonged release and specificity of GFAP led to the hypothesis for its use in stroke differentiation, as onset of ICH is typically rapid and any parenchymal injury should result in leakage of GFAP from astroglial cells.

A prospective study by Foerch et al. (93) involved 135 patients admitted to the hospital within 6 h of onset of stroke symptoms. Blood samples were obtained immediately upon admission, and patients were diagnosed with hemorrhagic or ischemic stroke based on CT or MRI results. Using an automated enzyme immunoassay, serum GFAP was detectable in 81% of patients with ICH, compared with only 5% of patients with ischemic stroke. In addition, serum GFAP concentrations were much higher in patients with ICH, with a mean recorded value of 111.6 ng/L vs 0.4 ng/L for patients with ischemic stroke ($P < 0.001$). At a cutoff of 2.9 ng/L, the diagnostic sensitivity of GFAP was 79% and specificity was 98% for differentiation between ICH and ischemic stroke ($P < 0.001$). In a subsequent study by the same group, the window of opportunity

for GFAP was determined to be 2–6 h after the onset of stroke symptoms to distinguish between ICH and ischemic stroke (94). The diagnostic accuracy within this time frame was between 83% and 88%. GFAP demonstrated low diagnostic sensitivity from 0 to 2 h of symptom onset, although only a small percentage of patients are triaged or admitted to the hospital within this timeframe.

A multicenter evaluation of S100B, neuron-specific enolase (NSE), GFAP, and activated protein C–protein C inhibitor complex (APC-PCI) demonstrated a significant ability of GFAP to distinguish ICH from ischemic stroke ($P = 0.005$) in a cohort of 97 stroke patients, a result not observed with S100B ($P = 0.13$), NSE ($P = 0.67$), or APC-PCI ($P = 0.84$) (95). Furthermore, combination of GFAP and APC-PCI with the NIHSS score yielded a diagnostic sensitivity and negative predictive value of 100%, allowing for rapid rule-out of ICH and potential earlier initiation of rtPA.

The only available assays for GFAP are enzyme immunoassays, which are currently not standardized. GFAP has demonstrated interesting preliminary clinical observations and appears to be a promising marker in hemorrhagic stroke, with potential for further use within a multimarker panel.

PARK7

The PARK7 (also known as DJ-1) protein was initially discovered as an oncogene (96) and later recognized as an autosomal recessive gene related to Parkinson disease (97). The mechanistic intricacies of PARK7 are unknown, but current hypotheses revolve around its reparative role in neurological oxidative stress damage and processes. Lescuyer et al. (98) identified PARK7 from a cohort of postmortem CSF proteins that were increased in comparison to antemortem CSF. Further analysis of plasma PARK7 concentrations using an ELISA demonstrated significant increases in stroke patients vs controls ($P < 0.001$), with increased concentrations occurring anywhere from 30 min to 3 h after the onset of symptoms (99). Using a PARK7 cutoff of 14.1 μg/L yielded a diagnostic sensitivity of 54% and specificity of 90%. Increases of PARK7 did not accurately distinguish between the type of stroke (ischemic, hemorrhagic, or TIA); thus an increased result would not allow for rapid initiation of rtPA without further imaging to rule out ICH. Further studies are needed to analyze and optimize the performance of PARK7 in an acute clinical setting.

NUCLEOSIDE DIPHOSPHATE KINASE A

Nucleoside diphosphate kinase A (NDKA) enzymes are responsible for catalyzing the exchange of phosphate between various nucleoside diphosphates.

Table 3. Miscellaneous biomarkers used in strategies for stroke diagnosis.		
Mechanism and biomarker	Physiological function	References
Inflammation		
CRP	Acute-phase reactant, part of innate immune response	Andersson et al. (110), Kaplan et al. (111)
VCAM-1[a]	Binds monocytes and lymphocytes	Lynch et al. (101)
MCP-1	Potent mononuclear cell produced by endothelial and smooth muscle cells	Reynolds et al. (100)
Dyslipidemia/endothelial damage		
ApoC1	Associated with LDL and VLDL, involved in plasma lipoprotein remodeling, inhibits CETP	Allard et al. (112)
ApoC3	Associated with VLDL, HDL and LDL; inhibits triglyceride hydrolysis by lipoprotein/hepatic lipase; interferes with normal endothelial function	Allard et al. (112)
BNP	Myocardial polypeptide with natriuretic, diuretic, and vasodilator activity	Makikallio et al. (113), Montaner et al. (114)
FABP	Cytoplasmic protein that modulates lipid signaling cascades; involved in fatty acid oxidation	Wunderlich et al. (115), Pelsers et al. (116)
Growth factors		
BDNF	Responsible for survival and maintenance of mature neurons	Reynolds et al. (100)
Endothelial damage		
MBP	Main proteolipid constituent of myelin, produced by oligodendroglia cells	Jauch et al. (80), Hill et al. (84)
NSE	Dimeric glycolytic isoenzyme in cytoplasm of neurons/neuroendocrine cells	Unden et al. (95), Anand and Stead (117)
Coagulation/fibrinolysis		
D-dimer	Fibrin degradation product, reflects a global activation of coagulation and fibrinolysis	Laskowitz et al. (103), Barber et al. (118)
von Willebrand factor	Multimeric adhesive glycoprotein important for platelet hemostatic interactions	Barber et al. (119), Folsom et al. (120)

[a] VCAM, vascular cellular adhesion molecule; MCP, monocyte chemotactic protein; Apo, apolipoprotein; FABP, fatty acid binding protein; BDNF, brain-derived neurotrophic factor; MBP, myelin basic protein.

NDKA is expressed in neurons and thought to be involved in the ischemic cascade following stroke. NDKA was identified and studied along with PARK7 (98) and analyzed with an ELISA by the same research group (99). Similar to PARK7, plasma NDKA concentrations increased early after symptom onset. The reported diagnostic sensitivity of NDKA improved slightly (67%) with comparable specificity (90%) to PARK7. Analogous to other biomarkers mentioned, the overall lack of diagnostic sensitivity precludes routine use of NDKA in stroke; however, the excellent specificity of NDKA may warrant further studies within a multimarker panel.

MISCELLANEOUS BIOMARKERS

Table 3 lists a number of other biomarkers that have been investigated in the context of stroke, either alone or in combination with other markers. In general, the biomarkers in Table 3 are rather nonspecific for stroke or, in fact, other physiological processes. Although there is not a wide body of supportive literature or data about the performance characteristics of these markers, they may play a role in stroke diagnosis, prognosis, or treatment in the future.

Role of Multimarker Panels

Currently there are no known individual biomarkers that can be targeted for routine use in the setting of acute stroke diagnosis, differentiation, or risk prediction. Multimarker panels have been developed and investigated in an attempt to improve the diagnostic sensitivity and specificity of individual biomarkers. For a multimarker panel to be successful, it must provide

additive information to the clinical diagnosis and yield rapid results, and the instrumentation needs to be easy to use and cost effective.

Reynolds et al. *(100)* screened a cohort of 223 stroke patients using a panel of markers that included S100B, B-type neurotrophic growth factor, von Willebrand factor, MMP-9, and monocyte chemotactic protein 1. They also recruited 214 normal controls and measured 50 serum biomarkers. Combining the 5 markers yielded a high diagnostic sensitivity (91%) and specificity (97%) for diagnosing acute ischemic stroke for specimens obtained within 12 h after symptom onset, compared to using any marker individually. A second study examined a panel of 26 markers in a cohort of 65 suspected ischemic stroke patients and 157 controls *(101)*. A diagnostic sensitivity and specificity of 90% was reported for stroke prediction when combining S100B, MMP-9, vascular cell adhesion molecule, and von Willebrand factor. In both of these studies, the majority of the control populations were age-matched subjects without any neurological symptoms, which are study limitations.

Laskowitz et al. *(102)* examined a panel of markers which included D-dimer, CRP, brain natriuretic peptide (BNP), MMP-9, and S100B in 130 patients. The cohort presented within 6 h of onset of symptoms and included individuals with suspected acute stroke and stroke mimics. There was a lower diagnostic sensitivity (81%) and specificity (70%) reported for prediction and diagnosis of ischemic stroke than the previous 2 studies using this panel of markers. The same group conducted a prospective, multicenter trial that evaluated the diagnostic ability of the same panel of markers, excluding CRP, using the Triage Stroke Panel on the triage meter point-of-care platform (Biosite Inc.) in more than 1100 patients presenting with suspicions of stroke *(103)*. The time from onset of symptoms to presentation was <24 h. The diagnostic sensitivity (86%) and specificity (37%) of the panel alone to distinguish between stroke and stroke mimics, while not optimal, may allow for early clinical intervention in some patients. The independent cutoff for each biomarker used in the panel to calculate the likelihood of stroke is unknown.

Excellent systematic reviews of stroke biomarkers for both prognosis and diagnosis of ischemic stroke were recently published by Whiteley and colleagues *(104, 105)*, and the diagnostic review examined 21 studies that evaluated 58 single biomarkers and 7 panels of several biomarkers. High diagnostic sensitivity or specificity was demonstrated for the majority of the biomarkers when used alone; however, there were major limitations to the study designs and reporting that prevented the recommendation of a specific biomarker for clinical use. Common study flaws noted in the re-

view included small study sizes, poor choice of reference standard and selection of the control population, unclear diagnostic cutpoints, and overall lack of analytical characterization and clinical validation of the proposed biomarkers *(104)*. None of the multimarker panel studies provided regression equations to determine stroke probability, and various cutoffs for the same biomarker were used. In addition, the collection time points were often outside the window of treatment opportunity. Several other miscellaneous biomarkers have been investigated in stroke, either alone or within a panel, although there is not a wide body of supportive literature or data about the performance characteristics of the markers (Table 3).

There is a huge potential for a group of robust biomarkers within a multimarker panel to rapidly improve on the initial triage process in stroke and positively impact medical, financial, and operational outcomes. However, there have been no studies to date which have comprehensively evaluated or simulated the financial aspects of a single vs multimarker approach in the setting of stroke. Point-of-care cardiac marker testing for acute myocardial infarction has shown monetary and operational benefits in multiple studies *(106–108)* but has yet to demonstrate an improvement in clinical outcomes *(109)*. Furthermore, accurate quantification of the potential impact and benefit of laboratory testing in almost any clinical situation is difficult because of complexities within the healthcare system and the inevitable intertwining of potential variables in the financial equations.

Conclusions

This review addresses the current state of stroke diagnosis and examines several potential biomarkers for use in risk stratification, prediction, and diagnosis. Clearly there is much work needed before promising biomarker candidates can be introduced into the clinical laboratory. Because expedient results are key, the primary focus for new biomarkers of stroke should be availability as close to the patient as possible. In theory, development of point-of-care assays has the potential to have the greatest impact on the treatment and management of stroke and should remain a priority in biomarker development. Any new biomarker must be shown to add independent information to clinical judgment and imaging modalities.

Future studies evaluating novel stroke biomarkers should answer questions that address their unique clinical contribution in the diagnosis, management, and risk prediction of stroke: has the patient had a stroke? Is the stroke of ischemic or hemorrhagic etiology? Are symptoms suggestive of additional intensive investigation or thrombolytic therapy? Is the patient at risk for stroke or

reoccurrence of cardiovascular events? Modern stroke diagnosis remains heavily reliant on clinical interpretation, and further translational research efforts toward discovery of stroke biomarkers have the possibility to greatly improve patient outcomes and quality of care.

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