

Biomarker Panels in Ischemic Stroke

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Biomarkers have been sought to improve the diagnosis of stroke and determine the cause of stroke. In acute settings distinguishing ischemic stroke from other neurological diseases can be challenging, particularly when symptoms are mild. Determining the cause of stroke can also be challenging and frequently remains unclear or even unknown based on current diagnostic investigations and classification features. Correct diagnosis of ischemic stroke and its causes is essential to optimally treat and prevent stroke. Just as neuroimaging, cardiac evaluation and arterial imaging are used in the diagnosis of stroke and determining its causes, molecular features in the form of proteins, RNA, metabolites, lipids, and other biomarkers may also have utility.

Biomarkers are currently used in stroke. In the American Heart Association/American Stroke Association stroke prevention guidelines, class Ia and IIb recommendations are made on the use of low-density lipoprotein-cholesterol and hemoglobin A1c (HbA1c).¹ Likewise, in the American College of Cardiology/American Heart Association cardiovascular disease risk assessment guidelines, class IIa and IIb recommendations are made for HbA1c, C-reactive protein, lipoprotein-associated phospholipase A2, and urinary albumin excretion.^{2,3} Other biomarkers have been well studied in stroke including natriuretic peptides, glial fibrillary acidic protein, S100b, neuron-specific enolase, myelin basic protein, interleukin-6, matrix metalloproteinase (MMP)-9, D-dimer, and fibrinogen.^{4,5} Despite considerable effort, a troponin-like biomarker to aid in the diagnosis of stroke has remained elusive. The reasons for this may relate in part to the fact that stroke is a heterogeneous disease with variability in infarct size, location, and cause. The blood–brain barrier has also been suggested to impede release of markers specific to brain injury (eg, neuron-specific enolase, glial fibrillary acidic protein, S100b). In addition, many biomarkers associated with ischemic stroke are not disease specific and have been associated with other acute brain injuries including intracerebral hemorrhage, subarachnoid hemorrhage, and traumatic brain injury.

Given the heterogeneity in ischemic stroke, a single biomarker may not be able to sufficiently reflect the underlying complexity. This has kindled interest in the use of biomarker panels. A biomarker panel is a group of markers that reflect different pathophysiological processes of a disease. In stroke such markers might provide information about atherosclerosis, thrombus formation, inflammation, oxidative stress, endothelial injury, blood–brain barrier disruption, and cerebral ischemia. A

common approach to improve classification is the use of multiple markers. For example, in the classification of fruit an orange can best be distinguished from other produce when multiple features are assessed such as shape, color, texture, and smell. Similarly in stroke, multiple markers combined in a biomarker panel may improve diagnostic sensitivity and specificity.

Biomarker panels may have several applications in stroke. They may be useful to differentiate ischemic stroke and transient ischemic attacks (TIAs) from hemorrhagic stroke and other stroke mimics. In such studies it will be important to determine whether the biomarker panel can add to neuroimaging diagnosis of stroke and potentially aid in the early diagnosis of brain ischemia. It should also be determined whether biomarker panels may be a significant diagnostic tool in settings where neuroimaging is not readily available or in patients where mild brain ischemic injury is not well visualized by MRI. Biomarker panels may also be useful to determine cause of stroke, particularly in patients where cause cannot be ascertained using current technology. Biomarker panels may also find applications to stratify risk of future stroke, provide prognostic information, or potentially identify patients most likely to benefit from stroke treatments such as reperfusion or hypothermia. In this summary, we discuss the different types of molecules that can be included in a biomarker panel, the methods to select which biomarkers to include in a panel, and the approaches to develop and assess prediction models developed from biomarker panels. Finally, we describe some of the initial studies of biomarker panels in ischemic stroke.

Molecules to Include in Biomarker Panels and Their Measurement

Ideally, a biomarker for stroke should be rapidly measured using a method that can be applied across a diverse range of clinical settings. It should be reproducible, reliable, and accurate. The assay should also be easy to interpret, cost effective, and importantly add to existing methods to diagnose ischemic stroke and determine etiology. These are demanding objectives to achieve. To date, >58 markers have been evaluated for the diagnosis of stroke, and several of these have been combined into biomarker panels^{4,6,7} (Table 1). Initial studies have also identified several biomarkers associated with cardioembolic, large vessel, and lacunar stroke (Table 2). However, with >250 000 proteins, in addition to 20 000 coding genes and an ever enlarging number

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Table 1. Biomarker Panels for the Diagnosis of Ischemic Stroke and Stroke Cause

Panel	Comparison	Marker Type	Assay Platform	Sample Size	Markers in Panel	Sensitivity/Specificity
22 gene panel ⁸	Ischemic stroke vs control	RNA, PBMCs	Microarray, Affymetrix U133A	40	ADM, APLP2, BST1, C1QR1, CD14, CD163, CD36, CSPG2, CYBB, DUSP1, ENTPD1, ETS2, FCGR1A, FLJ22662, FOS, IL13RA1, KIAA0146, LTA4H, NPL, PILRA, TLR2	78%/80%
18 gene panel ⁹	Ischemic stroke vs control	RNA, whole blood	Microarray, Affymetrix U133 Plus 2.0	60	ARG1, BCL6, CA4, CKAP4, ETS-2, HIST2H2AA, HOX1.11, F5, FPR1, LY96, MMP-9, NPL, PYGL, RNASE2, S100A9, S100A12, S100P, SLC16A6	89%/100%
9 gene panel ¹⁰	Ischemic stroke vs control	RNA, whole blood	Microarray, Illumina HumanRef-8v2 bead chip	64	ARG1, CA4, CCR7, CSPG2, IQGAP1, LY96, MMP-9, ORM1, S100A12	...
18 gene panel ¹¹	Ischemic stroke vs control	RNA, whole blood	Microarray, Affymetrix U133 Plus 2.0	237	ARG1, BCL6, CA4, CKAP4, ETS-2, HIST2H2AA, HOX1.11, F5, FPR1, LY96, MMP-9, NPL, PYGL, RNASE2, S100A9, S100A12, S100P, SLC16A6	93%/95%
6 protein panel ¹²	Ischemic+hemorrhagic stroke vs control	Protein, plasma	ELISA	1005	Caspase-3, Chimerin, D-dimer, MMP-9, Secretagogin, sRAGE	17%/98%
5 protein panel ¹³	Ischemic+hemorrhagic stroke vs control	Protein, plasma	ELISA	437	BNGF, MCP-1, MMP-9, S100B, vWF	92%/93%
4 protein panel ¹⁴	Ischemic+hemorrhagic stroke vs control	Protein, plasma	ELISA	946+343	BNP, D-dimer, MMP-9, S100B	86%/37%
4 protein panel ¹⁵	Ischemic stroke vs control	Protein, plasma	ELISA	222	MMP-9, S100B, VCAM1, vWF	90%/90%
4 protein panel ¹⁶	Ischemic stroke vs control	Protein, whole blood	Immunoassay	155	BNP, D-dimer, MMP-9, S100B	73%/72%
5 protein panel ¹⁷	Ischemic+hemorrhagic stroke vs control	Protein, plasma	Immunoassay	130	Eotaxin, EGFR, S100A12, Metalloproteinase inhibitor-4, Prolactin	90%/84%
2 protein panel ¹⁸	Cardioembolic vs noncardioembolic stroke	Protein, plasma	ELISA	707	D-dimer, BNP	87%/85%
40 gene panel ¹⁹	Large vessel vs cardioembolic stroke	RNA, whole blood	Microarray, Affymetrix U133 Plus 2.0	99	ADAMTSL4, AP3S2, ARHGEF12, ARHGEF5, BANK1, C16orf68, C19orf28, CD46, CHURC1, CLEC18A, COL13A1, EBF1, ENPP2, EXT2, FCRL1, FLJ40125, GRM5, GSTK1, HLA-DOA, IRF6, LHFP, LHFP, LOC284751, LRRC37A3, OOEP, P2RX5, PIK3C2B, PTPN20A, TFDP1, TMEM19, TSKS, ZNF185, ZNF254	100%/96%
41 gene panel ²⁰	Lacunar vs nonlacunar stroke	RNA, whole blood	Microarray, Affymetrix U133 Plus 2.0 Microarray	131	ALS2CR11, C18orf49, CALM1, CCDC114, CCDC78, CCL2, CCL3, CHML, FAM179A, FAM70B, FLJ13773, GBP4, GTF2H2, HLA-DQA1, HLA-DRB4, IL8, LAG3, LAIR2, LGR6, LRRC8B, MPZL3, OASL, PDXDC1, PROCR, PRSS23, QKI, RASEF, RUNX3, SCAND2, STK4, STX7, TGFBR3, TSEN54, TTC12, UBA7, UGCG, UTS2, VAPA	>90%/90%

BNGF indicates B-type neurotrophic growth factor; BNP, brain natriuretic peptide; CCR7, chemokine receptor 7; EGFR, epidermal growth factor receptor; IL, interleukin; MCP-1, monocyte chemoattractant protein-1; MMP-9, matrix metalloproteinase 9; S100, S100 calcium binding protein; sRAGE, soluble receptor for advanced glycation endproducts; VCAM1, vascular cell adhesion molecule; and vWF, von Willebrand factor.

Table 2. Biomarkers Associated With Cause of Ischemic Stroke

Biomarker	Cause of Stroke	Description of Biomarker
BNP ^{21,22}	Cardioembolic	Vasoactive peptide hormone
von Willebrand factor ^{23,24}	Cardioembolic	Glycoprotein
Interleukin-6 ^{25,26}	Cardioembolic, lacunar	Inflammatory cytokine
TNF- α ²⁵	Cardioembolic, lacunar	Inflammatory cytokine
D-dimer ^{18,27-29}	Cardioembolic, large vessel	Fibrin degradation product
C-reactive protein ^{30,31}	Cardioembolic, large vessel, lacunar	Acute phase protein
ICAM-1 ³²⁻³⁴	Lacunar, large vessel	Adhesion molecule
sRAGE ¹⁸	Lacunar, large vessel	Transmembrane receptor
Fibrinogen ^{31,35}	Large vessel	Glycoprotein
P-selectin ³⁶	Large vessel	Cell adhesion molecule
Adiponectin ³⁷	Large vessel	Adipose tissue hormone
Thrombomodulin ³⁴	Lacunar	Thrombin cofactor
RNA panel ^{19,20,38}	Cardioembolic, large vessel, lacunar	Nucleic acid

BNP indicates brain natriuretic peptide; ICAM-1, intracellular adhesion molecule-1; sRAGE, soluble receptor for advanced glycation end products; and TNF- α , tumor necrosis factor- α .

of noncoding genes, metabolites, and lipids, it is important to recognize that the molecular features of human stroke are still being determined and evaluated. Of the many molecules, those with optimal biomarker potential in stroke likely remain largely unknown. Efforts to define the molecular features of stroke are ongoing and support recommendations by the National Research Council of the US National Academy of Sciences to build a knowledge network and taxonomy of human disease.³⁹

Continued advances in technology are improving the ability to evaluate stroke at a molecular level. Increasingly, large-scale methods are being used to identify candidate molecules including screening of proteins, lipids, RNA, and metabolites. For proteins methods include ELISA, aptamer-based assays, mass spectrometry, and 2-dimensional gel electrophoresis. For RNA methods include reverse transcription polymerase chain reaction, microarray, sequencing, and nanostring technologies. Increasingly lipids and metabolites are also being assessed on a large-scale use mass spectrometry. These methods will identify novel markers and better define the biological pathways important to stroke and its causes. Although each marker on its own may not be unique to stroke or an individual cause of stroke, when several of these markers are assessed together in a panel, the ability to identify stroke or determine stroke cause may become possible.

Assays of proteins in plasma or serum have been a common approach to measure biomarkers in stroke and provide valuable insight in the development of stroke biomarkers.^{4,6,7} Variability in biomarker measurement, particularly between sites, has been identified. For example, in a meta-analysis of interleukin-6 in stroke and another meta-analysis of brain natriuretic peptide (BNP) in ischemic stroke, >1000-fold difference in mean protein concentration was observed between sites.^{40,41} Reducing such variability will be essential to replicate promising biomarkers and biomarker panels in stroke. Recommendations have been made by the National Institutes of Health/National Institute of Allergy and Infectious Diseases-sponsored External Quality Assurance Program Oversight Laboratory to reduce variability in immune-based

assays and improve the discovery of biomarkers.⁴² These include rigorous standardized methods of sample collection, sample type, storage, processing, and measurement in addition to quality control protocols to assess each step.

Biomarker Selection

How to best select markers for a biomarker panel remains an active field of investigation. Although a comprehensive summary is beyond the scope of this article, it is important to recognize different approaches exist to build prediction models and are important to the success of a biomarker panel.^{43,44} One approach is to use all markers that are significantly different in stroke or cause of stroke and combine them to form a predictive model. However, 1 marker often provides similar predictive information compared with another marker despite both being significantly different between the compared groups. To identify markers that combine well together as predictors, a variety of feature selection methods are used. These include forward selection, backward selection, or combining markers into families, clusters or networks based on pattern of expression and biological information.^{45,46}

The number of markers included in a biomarker panel can range widely. For example, a ratio of 2 markers (E6/E7 mRNA) is used in the Aptima assay for human papilloma virus in cervical cancer.⁴⁷ In contrast, prognostic biomarker panels used in breast cancer have as many as 97 markers (Mamaprint 70 markers, Oncotype 16 markers, Prosigna 50 markers, Endopredict 8 markers, MapQuant 97 markers, and Rotterdam signature 76 markers).⁴⁸

Prediction Model Development

Once markers are selected, there are a variety of methods to assemble them into a prediction model.⁴⁹ Often mathematical methods are used to develop prediction models such as nearest centroid, k-nearest neighbor, discriminate analysis, support vector machines, partial least squares, logistic regression, or random forests. Other methods have also been used to combine multiple markers into predictive models. For example, in

the Aptima assay for human papilloma virus in cervical cancer, a ratio of one marker to the other is used.⁴⁷ Another strategy is to evaluate the change in markers over time such as the change in troponin >8 hours in myocardial ischemia. A multistage approach can also be used whereby a biomarker panel selects a group of patients to whom another biomarker panel can be applied. In patients with cryptogenic stroke, we applied a multistage approach to classify stroke pathogenesis.³⁸ Cryptogenic strokes with a small deep infarct were initially predicted to be either lacunar or nonlacunar stroke. Those predicted to be nonlacunar were then predicted to be either arterial or cardioembolic. Using this method 58% of cryptogenic strokes were predicted to be cardioembolic, 18% arterial, 12% lacunar, and 12% remained unclassified.

Prediction Model Evaluation

When a panel of markers is identified and assembled into a prediction model, its predictive ability needs to be assessed. In general, the predictive ability of a model will always be better in the cohort of subjects from which the markers were derived and the model developed. To avoid this bias, evaluation of a model in a second cohort of subjects is important to assess predictive ability. Initial studies often do not have a second cohort of subjects and methods such as cross-validation or bootstrapping are used to estimate a model's predictive performance. However, these methods use the original cohort from which the model was derived; therefore, bias in favor of the prediction markers remains. Once a biomarker panel is validated, the model needs to be locked down and evaluated in a larger cohort to determine the clinical use. Recommendations on biomarker panel development, validation, and evaluation have been published.^{50,51} Adherence to such recommendations is important to ensure the robust development and translation of biomarker panels in stroke.

Biomarker Panels in Ischemic Stroke

In ischemic stroke, several biomarker panels have been evaluated for the diagnosis of stroke and determining stroke pathogenesis (Table 1). In patients with ischemic stroke, we have described a 40 marker panel to distinguish cardioembolic from large vessel stroke,¹⁹ and a separate 41 marker panel to distinguish lacunar from nonlacunar stroke.²⁰ These panels, derived from patients with known cause of stroke, have been used to predict the likely cause in patients with cryptogenic stroke.³⁸ Such panels offer the advantage of improved sensitivity and specificity. Biomarker panels have also been evaluated for the diagnosis of ischemic stroke. These include a panel of 5 proteins (MMP-9, B-type neutrotrophic growth factor, von Willebrand factor, monocyte chemotactic protein-1, and S100B),¹³ a panel of 4 proteins (MMP-9, brain natriuretic factor, D-dimer, and S100B),¹⁴ and a panel of 5 proteins (eotaxin, epidermal growth factor receptor, S100A12, metalloproteinase inhibitor-4, and prolactin).¹⁷ The combination of multiple markers in a panel has consistently demonstrated improved sensitivity and specificity to identify acute ischemic stroke compared with individual markers. Although none have provided sufficient evidence to demonstrate clinical use, the results support the concept of combining multiple markers into a panel. These studies are summarized below and in Table 1.

A panel of 5 protein markers (S100B, B-type neutrotrophic growth factor, von Willebrand factor, MMP-9, and monocyte chemotactic protein-1) was initially evaluated in 223 patients with acute stroke (82 ischemic, 103 hemorrhagic) compared with 214 healthy controls. It was able to distinguish stroke from controls with 92% sensitivity and 93% specificity.¹³ Samples were acquired from plasma within 6 hours of stroke onset and evaluated by ELISA. More than 50 proteins were initially evaluated and reduced to the 5 markers. The final model of the 5 markers was developed using logistic regression and evaluated in the derivation cohort.

A subsequent study evaluated a panel of 4 markers (BNP, D-dimer, MMP-9, and S100B) in 585 patients with acute stroke (293 ischemic, 95 hemorrhagic, 197 TIA) compared with 361 stroke mimics. The panel was able to distinguish stroke from controls with 86% sensitivity and 37% specificity.¹⁴ Samples were acquired from plasma within 24 hours of stroke onset and evaluated by ELISA. The model was developed using logistic regression and evaluated initially in the derivation cohort. A second cohort of 343 subjects (87 ischemic, 64 hemorrhagic, 40 TIA, and 152 stroke mimics) yielded similar discriminative characteristics. Of interest, the addition of 3 clinical variables (age, sex, atrial fibrillation) to the biomarker panel model did improve test performance slightly, although most diagnostic discrimination was achieved by the biomarkers.

In a study of 915 patients with acute stroke (776 ischemic, 139 hemorrhagic) and 90 stroke mimics, a panel of 6 markers (caspase-3, D-dimer, soluble receptor for advanced glycation end products, chimerin, secretagogin, and MMP-9) identified stroke with 98% sensitivity and 17% specificity.¹² Samples were acquired from plasma within 24 hours of stroke onset and evaluated by ELISA. The model was developed using logistic regression and evaluated in the derivation cohort. As the number of biomarkers included in the model increased, the ability to identify stroke was found to improve. Of interest, markers such as S100B, brain natriuretic peptide, and neurotrophin-3 were not significantly different between stroke and stroke mimics and did not add to the predictive models for stroke diagnosis.

In a study of 130 patients with acute stroke (57 ischemic, 32 hemorrhagic, and 41 TIA) and 37 stroke mimics, a panel of 5 markers (eotaxin, epidermal growth factor receptor, S100A12, metalloproteinase inhibitor-4, and prolactin) distinguished stroke from controls with 90% sensitivity and 84% specificity.¹⁷ Samples were acquired from plasma within 24 hours of stroke onset and evaluated by immunoassay through Astute Medical Inc. Levels of 262 markers were initially evaluated and reduced to 5 markers by *P* value <0.2 filter followed by stepwise selection. The final logistic regression model based on the 5 markers was evaluated by bootstrap analysis of the derivation cohort. Of note, although previous biomarkers associated with stroke (MMP-9, D-dimer, and BNP) were identified on univariate analysis, they were not found to be the best predictors and were eliminated from the prediction model. This suggests that it is important to evaluate a broad range of molecules involved in the biology of stroke to identify the best predictors.

BNP and D-dimer have also been evaluated as a panel to determine cause of ischemic stroke.¹⁸ In 707 ischemic strokes,

a BNP >76 pg/mL identified cardioembolic stroke with 68% specificity and 72% sensitivity, and D-dimer >0.96 µg/mL identified cardioembolic stroke with 64% specificity and 56% sensitivity. When combined, BNP and D-dimer identified cardioembolic stroke with 87% sensitivity and 85% specificity. Samples were acquired from plasma within 24 hours of stroke onset and evaluated by ELISA. Logistic regression analysis was used and prediction model evaluated on the derivation cohort. When the model was combined with history of cardiomyopathy, atrial fibrillation, and baseline National Institutes of Health Stroke Scale the sensitivity was 66% and specificity 91.3%. Among patients with stroke initially classified as cryptogenic but later found to have a cardioembolic source, 70% had elevated levels of either D-dimer or BNP.

Conclusions

The need to improve the diagnosis of stroke and cause of stroke has motivated the search for biomarkers. Although several markers have shown promise, as yet none have sufficient evidence to support use in clinical practice. Ischemic stroke is a heterogeneous disorder and a single biomarker may not be able to reflect this complexity. A biomarker panel may be able to better reflect the diverse pathophysiology involved in stroke and thereby distinguish ischemic stroke from hemorrhage, predict which TIAs proceed to stroke, and predict causes of stroke. Initial studies of biomarker panels indicate improved diagnostic sensitivity and specificity can be achieved in stroke relative to individual markers. However, efforts are needed to better define the molecular biology of stroke including determination of the involved proteins, RNA, metabolites, and lipids. Omic-based approaches are proving useful to identify novel markers relevant to stroke biology and biomarker development. As these markers are identified, assembling them into biomarker panels offers promise to achieve the rigorous requirements of a diagnostic clinical stroke biomarker.

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