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Biomarker panels in ischemic stroke

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Ischemic Stroke; Biomarkers; Panels; Stroke Etiology

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, ribonucleic acid (RNA), metabolites, lipids and other biomarkers may also have utility.

Biomarkers are currently used in stroke. In the AHA/ASA stroke prevention guidelines, class Ia and IIb recommendations are made regarding the use of LDL-C and HgA1c[1]. Likewise, in the ACC/AHA cardiovascular disease risk assessment guidelines, class IIa and IIb recommendations are made for HgA1c, 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 (GFAP), S100b, neuron specific enolase (NSE), myelin basic protein (MBP), 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. NSE, GFAP, 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

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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 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 if 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 used for stroke diagnosis and etiology determination. These are demanding objectives to achieve. To date, over 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 a number of biomarkers associated with cardioembolic, large vessel, and lacunar stroke (Table 2). However, with over 250 000 proteins, in addition to 20,000 coding genes and an ever enlarging number of non-coding 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 (NRC) 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 2D gel electrophoresis. For RNA

methods include RT-PCR, 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. Though 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 IL-6 in stroke and another meta-analysis of 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 NIH/NIAID sponsored External Quality Assurance Program Oversight Laboratory (EQAPOL) 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. Though 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, one marker often provides very similar predictive information compared to another marker despite both being significantly different between the compared groups. In order 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/or biological information[45, 46].

The number of markers included in a biomarker panel can range widely. For example, a ratio of two markers (E6/E7 mRNA) is used in the APTIVA assay for HPV 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, 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 use to combine multiple markers into predictive models. For example, in the APTIVA assay for

HPV 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 over 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 etiology[38]. Cryptogenic strokes with a small deep infarct were initially predicted to be either lacunar or non-lacunar stroke. Those predicted to be non-lacunar 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 clinical utility. Recommendations regarding 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, a number of biomarker panels have been evaluated for the diagnosis of stroke and determining stroke etiology (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 non-lacunar 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 (MMP9, BNGF, vWF, MCP-1, S100B)[13], a panel of 4 proteins (MMP9, 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 to individual markers. Though none have provided sufficient evidence to demonstrate clinical utility, 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 (BNGF), von Willebrand factor (vWF), MMP-9, monocyte chemotactic protein-1) was initially evaluated

in 223 patients with acute stroke (82 ischemic, 103 hemorrhagic) compared to 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. Over 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, MMP9, S100B) in 585 patients with acute stroke (293 ischemic, 95 hemorrhagic, 197 TIA) compared to 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, 152 stroke mimics) yielded similar discriminative characteristics. Of interest, the addition of three clinical variables (age, gender, atrial fibrillation) to the biomarker panel model did improve test performance slightly, though 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 (sRAGE), 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, 41 TIA) and 37 stroke mimics, a panel of 5 markers (eotaxin, epidermal growth factor receptor (EGFR), 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, though previous biomarkers associated with stroke (MMP-9, D-Dimer, 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>76pg/ml identified cardioembolic stroke with 68% specificity and 72% sensitivity, and D-dimer >0.96ug/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 NIHSS the sensitivity was 66% and specificity 91.3%. Among stroke patients 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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Potential Conflicts of Interest

FRS has received modest royalties from Ischemia Care.

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Table 1

Biomarkers 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, MMP9, 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, MMP9, 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, MMP9, 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, MMP9, S100B	86%/37%
4 Protein Panel[15]	Ischemic Stroke vs Control	Protein, Plasma	ELISA	222	MMP-9, S100B, VCAM, 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 Non-Lacunar 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,	>90%/90%

Panel	Comparison	Marker Type	Assay Platform	Sample Size	Markers in Panel	Sensitivity / Specificity
					PRSS23, QKI, RASEF, RUNX3, SCAND2, STK4, SYTX7, TGFBR3, TSEN54, TTC12, UBE	
					PRSS23, QKI, RASEF, RUNX3, SCAND2, STK4, SYTX7, TGFBR3, TSEN54, TTC12, UBE	
					PRSS23, QKI, RASEF, RUNX3, SCAND2, STK4, SYTX7, TGFBR3, TSEN54, TTC12, UBE	

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

Abbreviations: BNP, brain natriuretic peptide; ICAM-1; Intracellular adhesion molecule-1; RNA, ribonucleic acid; sRAGE, soluble Receptor for Advanced Glycation Endproducts; TNF-α, tumor necrosis factor alpha.