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Stroke. published online March 21, 2013;
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0039-2499. Online ISSN: 1524-4628

Original Contribution

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Background and Purpose—There is limited information about changes in metabolism during acute ischemic stroke. The identification of changes in circulating plasma metabolites during cerebral infarction may provide insight into disease pathogenesis and identify novel biomarkers.

Methods—We performed filament occlusion of the middle cerebral artery of Wistar rats and collected plasma and cerebrospinal fluid 2 hours after the onset of ischemia. Plasma samples from control and patients with acute stroke were also analyzed. All samples were examined using liquid chromatography followed by tandem mass spectrometry. Positively charged metabolites, including amino acids, nucleotides, and neurotransmitters, were quantified using electrospray ionization followed by scheduled multiple reaction monitoring.

Results—The concentrations of several metabolites were altered in the setting of cerebral ischemia. We detected a reduction in the branched chain amino acids (valine, leucine, isoleucine) in rat plasma, rat cerebrospinal fluid, and human plasma compared with respective controls (16%, 23%, and 17%, respectively; $P<0.01$ for each). In patients, lower branched chain amino acids levels also correlated with poor neurological outcome (modified Rankin Scale, 0–2 versus 3–6; $P=0.002$).

Conclusions—Branched chain amino acids are reduced in ischemic stroke, and the degree of reduction correlates with worse neurological outcome. Whether branched chain amino acids are in a causal pathway or are an epiphenomenon of ischemic stroke remains to be determined. **(*Stroke.* 2013;44:00-00.)**

Key Words: cerebrospinal fluid ■ liquid chromatography ■ mass spectrometry ■ metabolomics ■ transient ischemic attack stroke

The underlying pathogenesis of acute ischemic stroke remains poorly understood, with a paucity of biological insight translating into useful therapy in patients. Metabolomics is an emerging analytic technology for understanding disease pathogenesis that can be applied to both animal models and patient blood samples. It therefore represents an attractive translational tool to link the biology of model systems to the pathophysiology in patients. Using either nuclear magnetic resonance spectroscopy or mass spectrometry (MS),[1] metabolomics can measure numerous small metabolites simultaneously.[2] MS-based profiling methods include gas chromatography–MS and liquid chromatography coupled to MS, the most common of which is tandem MS (LC-MS/MS).[3] Approaches that use LC-MS/MS are increasingly used because of their sensitivity, flexibility, and quantitative capability for small molecule detection.[2]

Metabolomic profiling has found application in other forms of metabolic stress,[4] including intense exercise,[5] myocardial ischemia,[6] myocardial infarction,[7] and diabetes mellitus,[8–10] but little is known about metabolite changes in the setting of stroke. A common strategy used, in prior metabolomics studies, was to compare the metabolome within subjects, before and after the exposure. However, baseline blood sampling is not feasible in patients with acute stroke. We therefore sought to establish a metabolomic profile in an animal model of ischemic stroke in which baseline sampling is possible, and then integrate the findings with profiling in individuals with acute ischemic stroke. Using a rodent filament occlusion model, we first identified potential candidates whose plasma and cerebrospinal fluid (CFS) were altered. We then evaluated those candidates in an analogous patient cohort in which plasma samples were collected in the acute setting. We hypothesized that we could detect a specific pattern of circulating metabolites that would reflect the chain of metabolic events that occur during cerebral ischemia. Our goal was to apply this new systematic tool as a first step to better understand the biology and pathogenesis of acute ischemic stroke. In doing so, we also explored whether these candidates might serve as potential biomarkers for diagnosis or prognosis.[11–13]

Received December 5, 2012; final revision received February 22, 2013; accepted February 25, 2013.

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Costantino Iadecola, MD, was guest editor for this article.

The online-only Data Supplement is available with this article at http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.111.000397/-/DC1.

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Stroke **is available at http://stroke.ahajournals.org** **DOI: 10.1161/STROKEAHA.111.000397**

Methods

Animals

Adult male Wistar rats weighing 275 to 350 g were obtained from Charles River Laboratories (Wilmington, MA). Animals were housed with free access to food and water. The evening before surgery, animals were given nothing per oral to avoid the effect of dietary intake on circulating metabolites. Transient filament occlusion was performed using a 4-0 siliconized suture (Doccol Corp, Sharon, MA) according to standard methods (Methods in the online-only Data Supplement).[14,15] Approximately 250 μL of plasma was withdrawn at baseline and at 2 hours after ischemia onset.

CSF (≈50 μL) was collected from the cisterna magna at 2 hours after ischemia, using a 27-gauge winged needle set attached to a 1 cm^3 syringe.[16] Animals were allowed to recover, and at 24 hours after ischemia, brains were harvested for 2,3,5-triphenyltetrazolium chloride staining to assess the size of stroke.[17] All experiments were approved under an institutionally approved protocol in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Patients

We analyzed EDTA-containing plasma samples collected at a single center, as part of a prospective 2 center biomarker study of acute ischemic stroke (Specialized Programs of Translational Research in Acute Stroke [SPOTRIAS] Network). The SPOTRIAS biomarker study enrolled consecutive patients aged ≥18 years between January 2007 and April 2010, who presented to the Massachusetts General Hospital Emergency Department within 9 hours of symptom onset, with symptoms consistent with ischemic stroke (see Methods in the online-only Data Supplement for additional details of the cohort and the patient data and imaging collection). Ischemic stroke was defined as acute-onset focal neurological deficit with neuroimaging evidence of infarction, or symptom duration >24 hours in the setting of negative diffusion-weighted MRI. Transient ischemic attack was defined as resolution of neurological symptoms within 24 hours that were consistent with a vascular ischemic event (n=18). The designation of "not a stroke" was reserved for subjects with a negative diffusion-weighted MRI who also had an alternative diagnosis for neurological symptoms at discharge (n=14). All subjects or their healthcare proxy provided informed consent, and this study was approved by the local institutional review board.

We applied a case-control design to mirror the animal modeling experiments. We defined 3 groups from the SPOTRIAS biomarker cohort: control, mild, and severe stroke groups. Controls included all subjects with a final diagnosis of transient ischemic attack or absence of stroke (n=32). A similar sized group of mild ischemic stroke was selected from cardioembolic stroke subjects, and 22 sequential subjects with a National Institutes of Health Stroke Scale (NIHSS) ≥4 were used. We also selected sequential subjects with severe cardioembolic stroke, defined as those with an NIHSS ≥15 (n=30). All subjects or their healthcare proxy provided informed consent, and this study was approved by the local institutional review board.

High Performance Liquid Chromatography and Tandem MS

EDTA blood samples were collected and immediately centrifuged to separate cellular material. Aliquots of plasma supernatant were frozen on dry ice and stored at −80°C until analysis. Plasma samples (10 μL) were deproteinized with 90 μL acetonitrile/methanol (3:1; v/v) containing internal standards (valine-d8 [Sigma-Aldrich] and phenylalanine-d8 [Cambridge Isotope Laboratories]). After centrifugation, the extracts were subjected to normal phase hydrophilic interaction chromatography. The chromatography system consisted high throughput screen prep and load autosampler (Leap Technologies, Carrboro, NC) connected to a high performance liquid chromatography pump (1200 Series, Agilent, Santa Clara, CA). MS data were acquired using a 4000 QTRAP triple quadrupole mass spectrometer (Applied Biosystems/Sciex, Framingham, MA) equipped with an electrospray ionization source. Positively charged amino acids, nucleotides, and neurotransmitters were selected for targeted MS/MS analysis using selected multiple reaction monitoring conditions determined previously using reference standards.[6,7]

A total of 68 endogenous metabolites were monitored and detected for each sample. The metabolites were selected on the basis of a broad representation of diverse metabolic pathways as possible, balanced against compatibility with the chromatography and MS ionization method. Deuterated internal standards (valine-d8 and phenylalanine-d8, Cambridge Isotope Laboratories (Andover, MA)) were included in each sample to monitor for quality control. Any sample with internal standard values ≥2 SD were excluded from peak integration and further analysis. In addition, pooled plasma samples were interspersed within each analytic run at standardized intervals, enabling the monitoring and correction for temporal drift in MS performance. Each of these samples were prepared, extracted, and processed as separate 10 μL aliquots from a larger pool of normal human plasma. Replicate injections of pooled plasma demonstrated that 50% of the analytes had a coefficient of variation ≤5% (including the branched chain amino acids [BCAA]), 69% of the analytes had a coefficient of variation ≤10%, and 91% had coefficient of variation ≤20%, which is consistent with prior studies.[9]

Statistical Analysis

Univariate Analysis

Differences in clinical and laboratory continuous variables were compared using Student *t* test or Mann–Whitney test, as appropriate. Categorical variables were compared using Fisher exact test. For the metabolite analysis in the animal samples, we used an uncorrected *P* value threshold of 0.05, using Mann–Whitney or Student *t* test, depending on data normality. In this exploratory phase, no correction for multiple comparisons was made.

In the human cohort analysis, we used a similar approach to our prior studies[5] and applied the Benjamini–Hochberg procedure[18] to limit the false discovery rate to q<0.1, which corresponded to a threshold of $P<0.015$. This would be expected to yield ≈1 false-positive discovery in 68 metabolites analyzed, assuming independent hypotheses. Moreover, this threshold also approximates the Bonferroni correction of the combined probability between the discovery cohort ($P<0.05$) and the human validation cohort ($P<0.015$) (ie, $0.05\times0.015=7.5\times10^{-4}$, whereas Bonferroni correction=$0.05/68=7.4\times10^{-4}$). Although many metabolites were associated with predefined groups (eg, amino acids, tryptophan derivatives, nucleotide metabolites, etc.), this is a conservative estimate because the number of independent tests was substantially lower than the nominal ones. Statistical analyses were performed using the STATA statistical software (release 12) or JMP 10 Pro (SAS Institute, Cary, NC).

Multivariate Analysis

To uncover the multivariate structure within the human data set, we performed principal component analysis and partial least-squares discriminant analysis using MetaboAnalyst 2.0 (Edmonton, BC, Canada).[19] Because each method provides slightly different insight into high-dimensional data, we performed to highlight the metabolites in common (see Methods in the online-only Data Supplement for further details).

Results

Using LC-MS/MS, we first examined serial blood samples after filament occlusion in a rat model of ischemic stroke. We collected plasma at baseline and 2 hours after ischemia, as well as CSF at the 2-hour time point. In pilot experiments, the placement of a laser Doppler flowmetry probe led to poor recovery of CSF (data not shown). Exploiting the variability in stroke volume that would occur in the absence of Doppler flowmetry, we designed our experiment as a comparison among sham, small stroke, and large stroke animal cohorts. Of 23 animals, 2 died acutely and the volume of infarct could

not be determined. Another animal assigned to the middle cerebral artery occlusion group had no infarction at 24 hours and was therefore excluded. The remaining 20 animals were included in the analysis: 7 sham-operated animals, 6 animals with small infarction (stroke volume 9±5%), and 7 animals with large infarction (stroke volume 29±5%; Table I in the online-only Data Supplement).

We measured a total of 68 metabolites in baseline and 2-hour follow-up plasma samples, and results were analyzed as a percent change from baseline, which adjusts for within animal variation. To eliminate any nonspecific effects of the operative technique, we compared percent metabolite changes in stroke animals to those in sham-operated animals. From baseline to 2 hours after stroke, there was a significant decrease in the concentration of BCAA leucine, isoleucine, and valine in the large stroke group (P=0.003, 0.01, and 0.04, respectively). BCAA are coordinately regulated, and the levels change in conjunction with each other.[20] Accordingly, a composite measurement of the BCAA showed a 16±6% decrease in large stroke ($P=1\times10^{-5}$; Figure 1D) and a nonsignificant trend in small stroke. Several other metabolites were altered in a dose-dependent manner in small and large stroke. These included stepwise increases in xanthosine (+57%; P<0.001), carnosine (+71%; P<0.005), and glutamate (+40%; P=0.01), and decreases in niacinamide (−31%; P=0.02) and phenylalanine (−18%; P<0.01) relative to sham-operated animals.

We also measured the same metabolites in the CSF obtained through cisterna magna puncture at 2 hours after onset of ischemia. Because the concentration of most metabolites in CSF is lower than in plasma, we excluded any CSF samples with visible blood contamination (Methods and Figure I in the online-only Data Supplement). Figure 2A through 2D shows that the individual BCAAs had a consistent trend toward a decrease (leucine −21%, P=0.06; isoleucine −23%, P=0.14; valine −22%, P=0.11). Moreover, a composite of BCAA demonstrated a decrease of 23±9% compared with sham CSF (n=5 for each group; P<0.005). Other significantly altered CSF metabolites included an accumulation of xanthosine (102%, P=0.01) and lysine (18%, P=0.02).

On the basis of the animal studies, concordant metabolite changes between plasma and CSF included valine, leucine, isoleucine, and xanthosine. We next evaluated whether these candidate metabolites were altered in the plasma of patients with acute stroke to determine whether these metabolite changes represented a common alteration. We obtained plasma samples from a cohort of patients in whom blood was collected acutely, shortly after presentation to the emergency department. We selected a subset of subjects to coincide with the animal modeling design, which included a control group (patients with a diagnosis of transient ischemic attack or absence of stroke), a group with mild stroke (patients with an NIHSS 4–5), and a severe stroke group (NIHSS 15–19). To limit potential heterogeneity, we focused on subjects with a cardioembolic cause of stroke. The clinical characteristics of the cohort are listed in Table 1. As would be expected, the stroke group had an older age and higher rates of atrial fibrillation compared with the control group. In addition, the large stroke group had a higher acute stroke volume, higher acute NIHSS, and worse 3-month neurological outcome as compared with the small or control groups.

We analyzed plasma samples obtained within 6±2 hours from the last seen well time, using our metabolomics method. Heat map correlation analysis confirmed a close association of the BCAAs (Figure 3, top right), consistent with the animal modeling data and with the known coordinated metabolism of these amino acids.[20] Analysis of individual metabolites showed that leucine, isoleucine, and valine were all decreased in stroke compared with control, and to a greater extent in large compared with small stroke (P<0.01 for each; Figure 4). Similarly the composite BCAA score demonstrated a 9±17%

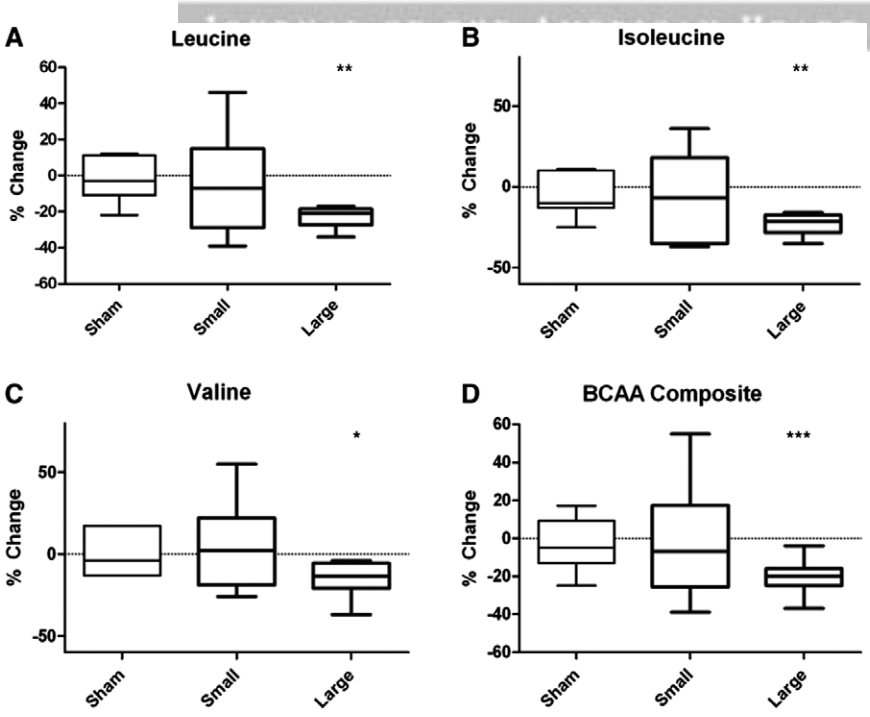

Figure 1. A–D, Rats subjected to filament occlusion of the middle cerebral artery had plasma collected at baseline (just before filament occlusion) and 2 hours after stroke. The concentration of branched chain amino acids (BCAA) were diminished from baseline to 2 hours after stroke. *P<0.05, **P<0.01, and *** P<0.001.

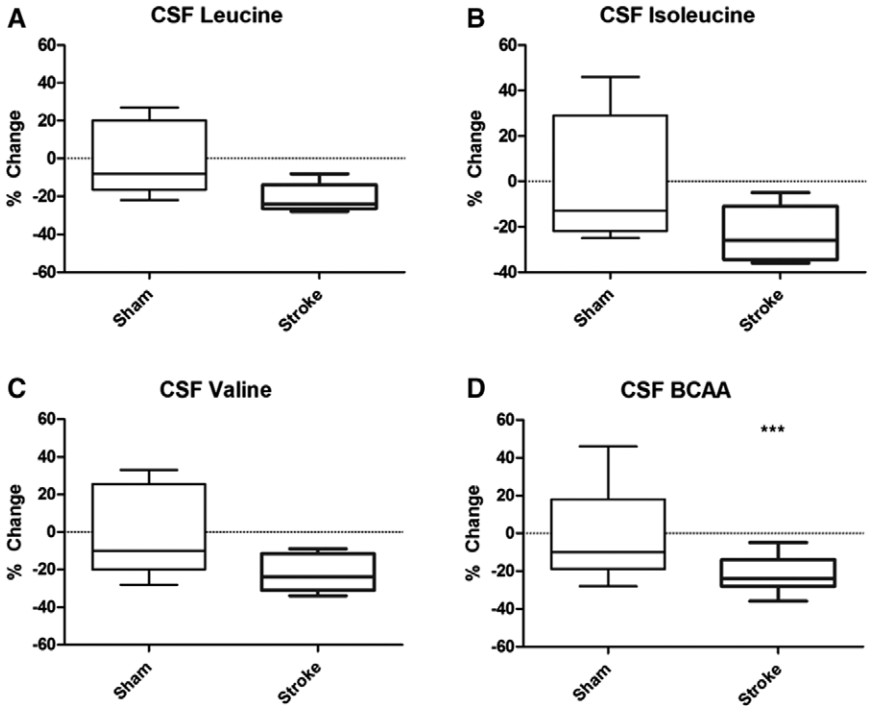

Figure 2. A–D, Change in branched chain amino acids (BCAA) in cerebrospinal fluid (CSF) from baseline to 2 hours after stroke (n=5 for each group). Each individual BCAA showed a trend toward a decrease, P=0.06, 0.14, and 0.11, respectively, whereas the composite BCAA, xanthosine and lysine were significant, $^{*}P$<0.01, $^{**}P$<0.01.

decrease in small stroke (P=0.03) and a 17±23% decrease in large stroke (P=1.1×10^{-5}). Table 2 provides a complete list of all metabolites that were altered in the setting of ischemia when compared with control patients. In addition to novel metabolites, we found that glucose showed a significant increase in stroke compared with control, which is concordant with the well-described phenomenon of acute stress hyperglycemia.[21–23]

To further simplify the high-dimensional metabolomics data, we next performed principal component analysis. This approach consolidates data into fewer metabolite clusters, which maximally explain the variance in the data.[19] Intriguingly, the first principal component (principal component, which explained 20% variance in the data; Figure II in the online-only Data Supplement for score and loading plots) contained the BCAA metabolites. In addition to leucine, valine, and isoleucine, the first principal component also included tyrosine, lysine, and methionine. Comparing the individual subjects' scores, the first principal component also distinguished cases from controls (P=0.020 comparing control versus all stroke and P=0.011 for control versus large stroke).

Next, we performed partial least-squares discriminant analysis, which is a method of supervised classification that is designed to highlight metabolite differences between cases and controls. This technique is commonly used in metabolomics studies for biomarker discovery because it emphasizes the distinction between the 2 classes.[19] The metabolites that contributed the greatest discrimination between stroke and controls were similar to our univariate analysis presented in Table 2. These included the BCAAs, carnitine, threonine, histidine, and glucose (Figure III in the online-only Data Supplement). Validation of the model was confirmed using cross-validation and permutation testing (P<0.01; Figure III in the online-only Data Supplement).[19,24]

Having confirmed that BCAA were altered acutely in stroke, we next explored its association with imaging and clinical measures. Because the magnitude of BCAA change seemed to correlate with size of stroke in the animal model, we evaluated the correlation between BCAA and diffusion-weighted volume in the patient cohort. There was a nonsignificant trend in association between admission infarct volume and BCAA (r=–0.18; P=0.11). On the contrary, a lower concentration of BCAA was associated with increased age (r=–0.26; P=0.02), female sex (P<0.001), and worse outcome at 3 months (modified Rankin Scale, 3–6; P=0.002). Because age and sex are also recognized predictors of worse neurological outcome,[25,26] we explored whether BCAA predicted outcome independently of age and sex. Although the

Table 1. Clinical Characteristics of the Stroke Cohort

	TIA (N=32)	Stroke (N=52)	*P* Value
Female, N, %	16 (50%)	22 (42%)	0.51
Age±SD, y	66±16	75±10	<0.01
Admission temp, °F±SD	98.3±0.7	98.2±1.1	0.72
CAD, N, %	9 (28%)	17 (33%)	0.81
HTN, N, %	25 (75%)	45 (87%)	0.37
DM2, N, %	11 (34%)	12 (23%)	0.32
HL, N, %	15 (47%)	23 (44%)	0.83
Afib, N, %	5 (16%)	33 (63%)	<0.001
		Small	Large	
NIHSS, median [IQR]	3 [1, 8]	4 [4, 5]	17 [15, 19]	<0.001
DWI volume, median [IQR]	0 [0, 3]	3 [1, 15]	25 [11, 59]	<0.001
3 Months mRS, 0–2, %	23 (79%)	11 (65%)	5 (23%)	<0.001

Afib indicates atrial fibrillation; CAD, coronary artery disease; DM2, diabetes mellitus 2; DWI, diffusion-weighted imaging; HL, hyperlipidemia; HTN, hypertension; IQR, interquartile range; mRS, modified Rankin Scale; NIHSS, National Institutes of Health Stroke Scale; TIA, transient ischemic attack.

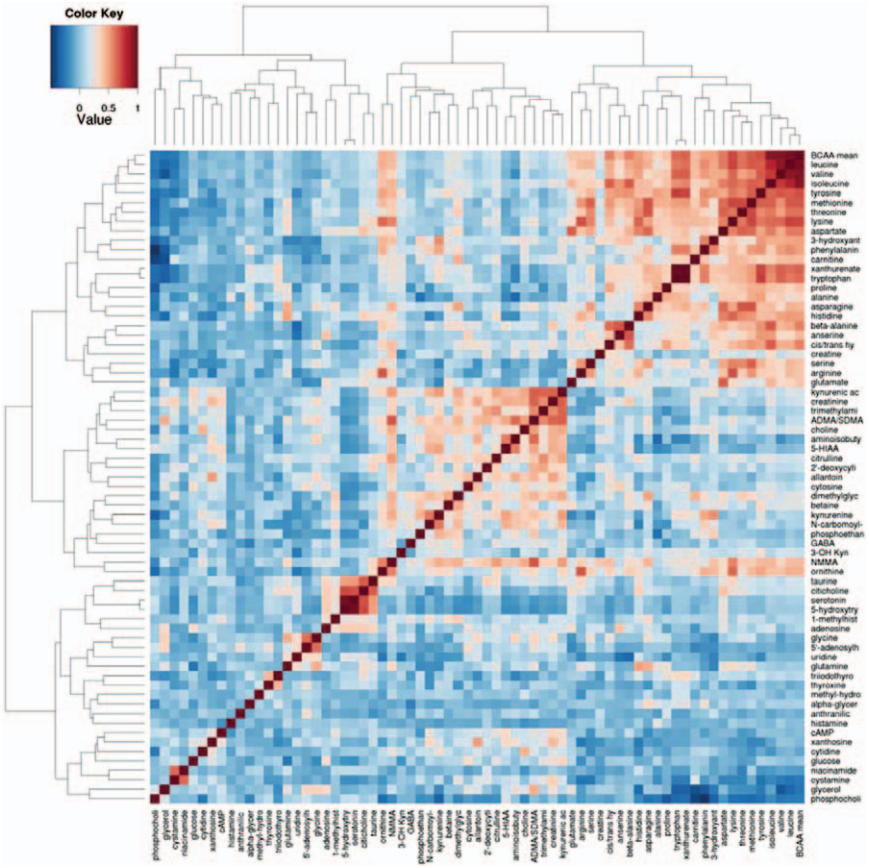

Figure 3. Heat map representation of metabolites highlights the tight correlation of the branched chain amino acid (BCAA), which are located in the top right. The heat map is generated from 52 patients with acute stroke who had blood samples drawn at 6±2 hours from the last seen well time. Analytes that are positively correlated are represented in red, whereas compounds inversely correlated are represented in blue. ADMA indicates asymmetric dimethylarginine; GABA, gamma aminobutyric acid; HIAA,5-hydroxyindoleacetic acid; and SDMA, symmetric dimethylarginine.

cohort was limited in size and stratified on the basis of stroke severity, we performed exploratory multivariable logistic regression and found that BCAA remained an independent predictor of outcome (P=0.04) after adjusting for age and sex.

Discussion

Using metabolomics, we have identified specific circulating metabolites that are altered in the setting of cerebral infarction. On the basis of our systematic analysis in a well-controlled

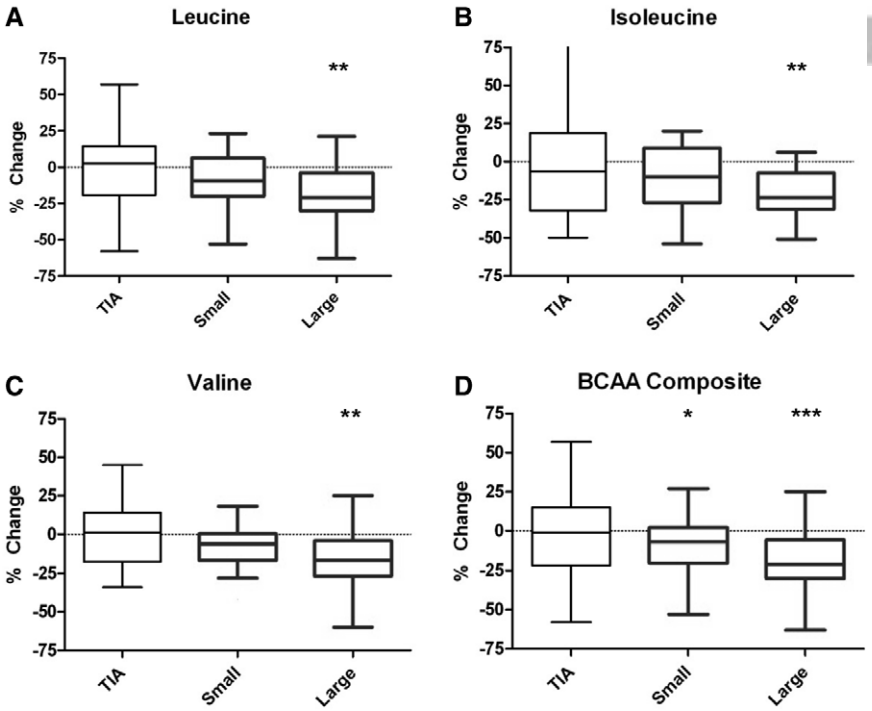

Figure 4. A–D, The concentration of plasma branched chain amino acids (BCAA) in patients with stroke is reduced when compared with control subjects at the time of acute presentation. *P<0.05, **P<0.01, and ***P<0.001.

Table 2. All Metabolites Significantly Changed in Human Stroke Subjects Compared With Control

Metabolite	Fold Change In Stroke	*P* Value	BH Procedure, Q<0.1
Carnitine	0.89	0.001	0.0015
Threonine	0.80	0.002	0.0029
Histidine	0.83	0.003	0.0044
Glucose	1.42	0.0057	0.0059
Valine	0.88	0.007	0.0074
BCAA mean	0.86	0.008	0.0088
Methionine	0.82	0.009	0.0103
Leucine	0.86	0.009	0.0117
Glycine	0.82	0.0131	0.0132
Proline	0.89	0.017	0.0147
Lysine	0.86	0.025	0.0162
Cysteamine	0.56	0.027	0.0177
Isoleucine	0.85	0.028	0.0191
Uridine	0.81	0.033	0.0258
5′-Adenosylhomocysteine	0.84	0.036	0.0221
Creatinine	0.89	0.039	0.0235
N-carbamoyl-β-alanine	1.33	0.041	0.0250
cis/trans Hydroxyproline	0.73	0.041	0.0265
Asparagine	0.89	0.043	0.0279

The false discovery threshold based on the Benjamini–Hochberg (BH) procedure[18] is indicated by the bold line. For completeness, additional metabolites that exceed this threshold, but with an uncorrected *P*<0.05, are listed below the bold line.

BCAA indicates branched chain amino acids.

animal model and linking those findings to patient samples in the acute setting, we have identified a small and interrelated subset of metabolites. Our data demonstrate a reduction in the concentration of BCAAs that associates with stroke severity and worse neurological outcome. Although our data do not point to an underlying biological mechanism, they focus future experiments on investigating candidate pathways that relate to BCAA. The notion that BCAA play an important role in the metabolic response to disease is supported by evidence of its alteration in other illnesses. For example, BCAA is reduced in critical illnesses, such as sepsis, trauma, and burn injury.[27–29] BCAA is also associated with the risk of incident diabetes mellitus[9] and can induce insulin resistance,[8] further suggesting a role in metabolic homeostasis. Perhaps, most interestingly, BCAA are altered also in heart disease,[30] suggesting that these amino acids play a critical role in bioenergetic homeostasis. Whether BCAA represent a novel link between cardiovascular and cardioembolic cerebrovascular diseases requires further investigation.

In addition to their potential role in systemic disease states, BCAA also serve a unique role in the brain.[31,32] For example, BCAA are integral to the glutamate/glutamine cycle between astrocytes and neurons, which is critical for the efficient uptake of glutamate during excitatory neuronal signaling.[31] Intriguingly, inhibition of the first step of BCAA catabolism with gabapentin reduces brain glutamate concentration.[31] Gabapentin has been reported to reduce stroke volume in a rodent model,[33] and 1 possibility is that it may do so by limiting glutamate concentration and subsequent excitotoxicity. Although our rodent data showed an accumulation of glutamate, we did not detect a similar change in the patients. Whether this reflects inadequate power or greater complexity in the human cohort requires further study. Alternatively, the reduction in BCAA level may reflect a metabolic pathway leading to consumption or sequestration in a tissue compartment other than blood or CSF. BCAA are also known to have roles in protein metabolism and in catabolic energy metabolism.[20] These putative mechanisms are not mutually exclusive, and, indeed, systemic BCAA levels have been shown to influence brain neurotransmitter levels.[32] Nevertheless, our data raise the possibility that manipulation of BCAA may influence outcome. Future studies that focus on whether BCAA are causally related to cerebral ischemia, such as through supplementation and pharmacological inhibition, will help determine whether BCAA holds promise as a therapeutic target.

Our analysis in rodents and patients identified additional candidate metabolites, which were not shared in common between the 2 (Table 2 and Results). The similarities and differences between rodent model systems and patients are an area of substantial importance for translational therapy. Metabolomics is a technique that allows direct comparisons between the model systems and patients in a way that was not previously available. Although our findings with BCAA highlight that there are similar biological pathways in rodents and patients, the differences may offer some caution. Nevertheless, our data point to one approach to explore these similarities and differences systemically, both of which are important for novel target discovery. There is little prior metabolomics analysis of stroke, with the exception of an nuclear magnetic resonance–based study in a cohort of lacunar stroke subjects,[34] which analyzed blood samples collected within 72 hours of stroke onset. Of the overlapping metabolites in common with our method, valine was diminished in lacunar stroke, although leucine and isoleucine were not.[34] The apparent differences may reflect the increased sensitivity of LC-MS/MS compared with nuclear magnetic resonance, differing metabolomes based on stroke subtype, differences in control selection, and potentially in the timing of the blood draw.

Our study has several strengths. We used a carefully controlled model system to establish a metabolite profile and then compared it to a well-phenotyped patient cohort. We used a metabolomics technique that is well validated and possesses excellent quantitative capability and reproducibility. The patient samples were obtained in the hyperacute phase and compared with a control group of stroke mimics. However, there are several limitations to our analysis. We used a targeted metabolomics approach, which identifies a limited set of metabolites rather than a comprehensive list of known and unknown peaks. It is therefore possible that additional metabolite changes occur that we cannot detect with our current method. LC-MS/MS–based metabolomics also has limited throughput capability. Nevertheless, we have selected key sentinel metabolites that are central to several important biochemical pathways, including amino acids, nucleotides, and selected neurotransmitters. Although our data point toward a key role for BCAA in stroke, our correlation and

multivariate regression must be interpreted with caution in a small patient cohort. Most importantly, validation in a larger cohort that includes all stroke subtypes with a broad range of stroke severity will be necessary to confirm our findings and determine whether BCAA holds promise as a clinically useful biomarker or a therapeutic target.

Sources of Funding

This study was supported by the Clinical Investigator Training Program: Beth Israel Deaconess Medical Center, Harvard Medical School, in collaboration with Pfizer Inc and Merck and Co (Dr Kimberly), by National Institute of Health (NIH) K23NS076597 (Dr Kimberly), NIH 5P50NS051343-07 (Dr Furie), and NIH R01HL096738 and R01HL098280 (Dr Gerstzen).

Disclosures

Dr Kimberly discloses a research grant (National Institute of Health [NIH]; significant) and Dr Gerszten discloses a research grant (NIH; significant).

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SUPPLEMENTAL MATERIAL

Metabolite Profiling Identifies a Branched Chain Amino Acid Signature in Acute Cardioembolic Stroke

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Supplemental Methods

MCAO surgical model

Following general anesthesia with 2% isoflurane in 30% oxygen and 70% air, permanent focal cerebral ischemia was induced using the standard intraluminal occlusion method. The rectal temperature was maintained at 37°C through a thermostat controlled heating pad. Continuous physiological monitoring was performed using PowerLab 8/30 instrumentation (AD Instruments, Milford, MA) equipped with a blood pressure bridge amplifier and thermostat connected to the homeothermic controller and heating plate (Physitemp, Clifton, NJ). A femoral artery catheter was placed using PE-50 tubing and plasma samples were collected just prior to filament occlusion and at 2 hours after occlusion. Filament occlusion was performed using a 4-0 siliconized suture (Doccol Corp, Sharon, MA) according to standard methods [1, 2]. Briefly, after exposure of the right carotid bifurcation and suturing of the common carotid (CCA) and distal external carotid (ECA) arteries, a siliconized suture was advanced to the level of the middle cerebral artery through an arteriotomy in the proximal ECA. Placement was confirmed by the slight resistance that was encountered at approximately 19-20 mm from the arteriotomy incision.

In order to best determine the stroke volume at the time of blood and CSF collection, we withdrew the filament shortly after the final sampling at 2-hours ischemia time. Because TTC staining is performed at 24 hours after ischemia, we were concerned that a permanent occlusion would lead to continued infarction and would misrepresent the size of stroke at the time of sample collection. By removing the filament just after the 2-hour collection time point, this created a "snapshot" of the degree of ischemia for each individual animal.

CSF blood contamination analysis

Because CSF contains lower abundance of many metabolites compared to blood, we evaluated the effect of blood contamination on metabolites measured by our metabolomics method. Since a traumatic tap can lead to blood contamination of the CSF, and the level of most small molecules is lower in CSF than plasma, we first evaluated the effect of blood contamination on metabolite levels. CSF samples with visible evidence of blood contamination were compared to samples without blood. The median level of all metabolites was 285% greater in samples grossly contaminated with blood at the time of CSF collection (IQR 127% to 602%; $p=8.1x10^{-10}$; supplemental figure 1). Given the magnitude of confounding effect of traumatic blood contamination in CSF, we excluded any CSF samples that were grossly contaminated from further analysis.

Patient Data

The SPOTRIAS biomarker study enrolled consecutive patients ≥18 years between January 2007 and April 2010, who presented to the Massachusetts General Hospital Emergency Department within 9 hours of symptom onset, with symptoms consistent with ischemic stroke. If the last seen well time was unknown, stroke onset was defined as the halfway point between the last seen well and first discovered times. Subjects with active infection (body temperature >101°F or WBC>15,000/mm^3), dialysis dependent renal failure or end-stage hepatic dysfunction, active metastatic malignancy at the time of stroke, myocardial infarction (MI), or major thromboembolic event or surgery within 30 days were excluded. In order to avoid any effect of the extremes of age, subjects >90 years of age were excluded from metabolite analysis (N=3 for mild stroke and N=3 for severe stroke).

The selection of this cohort was based on the potential ability to distinguish acute stroke patients from stroke mimics in the emergency department. The identity of a metabolite signature unique to stroke patients would be an important step towards identifying novel biomarkers for diagnosis. Because the control and stroke patients were collected as part of a carefully designed, prospective biomarker cohort, this ensured the homogeneity in the timing of collection, the method of collection (i.e., serum versus plasma), and its processing and storage. For example, this designed allowed us to minimize the potential effect of nutritional status. It is standard of care at our hospital that TIA and stroke subjects remain NPO for the first 24 hours after presentation. Since all cases and controls were NPO for at least 6 hours prior to the research blood draw, the contribution of the last meal to the metabolite profile would be minimal.

Demographic and clinical data were collected for each patient at baseline through direct interview or chart abstraction. Common vascular risk factors, medications, and routine laboratory data were assessed at baseline. Stroke severity (NIHSS) was obtained at baseline and functional outcome (modified Rankin Scale [mRS] score) was assessed by telephone or direct in-person interview at 3 months. Stroke subtype was assigned based on the Causative Classification of Stroke System (CCS) criteria [3-5].

Neuroimaging Analysis

Magnetic resonance imaging (MRI) was performed on all subjects at admission as part of the routine clinical stroke work-up. In this analysis, diffusion-weighted images (DWI) performed at the time of plasma sampling were used to assess baseline infarct volume. Imaging analyses were performed by trained readers blinded to all clinical data. Volumetric infarct data was collected using a validated semi-automated protocol where the intra-class correlation coefficient

was 0.99 [6]. Because infarct volumes are skewed [7, 8], data was logarithmically transformed prior to analysis.

HPLC-mass spectrometry conditions

HILIC chromatography was performed with a 150mm x 2.1mm Atlantis HILIC column (Waters Corp.). Whereas unbiased methods can identify a large array of peaks, the identity of those peaks may be ambiguous [9]. Therefore, we focused on a less comprehensive but more specific method where analytes are individually validated and confirmed upon addition to the method, which has been used by our laboratory for prior cardiometabolic metabolomic analyses [10-12]. Formic Acid, ammonium acetate, LC/MS grade solvents were obtained from Sigma-Aldrich. Mobile phase A was 99.5:0.5 (v/v) acetonitrile/formic acid and mobile phase B was 94.5:5:0.5 (v/v/v) 10 mM ammonium formate/acetonitrile/formic acid. Elution conditions were 95% mobile phase A for 5 min, then a linear gradient to 80% mobile phase B over 10 min, followed by a return to 95% mobile phase A over 2 min and 95% A for 15 min.

Following scheduled multiple reaction monitoring, the quantification of each metabolite was performed by integrating peak areas of the daughter ion using MultiQuant software (Version 2.1, Applied Biosystems/Sciex). All metabolite peaks were manually reviewed for peak quality in a blinded manner prior to statistical analysis.

Multivariate PCA and PLS-DA analysis

For principal component analysis (PCA), component scores for each individual subject in the dataset were calculated using the standardized scoring coefficients (consisting of a weighted sum of the values of the standardized metabolites within that component, weighted on the

component loading calculated for each individual metabolite). Metabolites with a component loading of > 0.2 or < -0.2 were identified as contributing to a given component. Because component scores account for the maximum data variance but not necessarily the discrimination between cases and controls, component scores were then used to assess the significance of difference in stroke versus control via the Mann-Whitney U test.

Partial least squares-discriminant analysis (PLS-DA) was performed to identify the metabolites that accounted for the greatest distinction between cases and controls. The PLS-DA model was constructed using 10-fold cross-validation and model quality was assessed using R^2 and Q^2 measures [13], which assess goodness of fit and goodness of prediction, respectively. Further validation was performed using permutation testing that is commonly employed for metabolomic studies [14], confirming the significance of the model in distinguishing a stroke-specific profile. Variable importance in projection plots were generated to graphically depict which metabolites were most important in the discrimination between cases and controls.

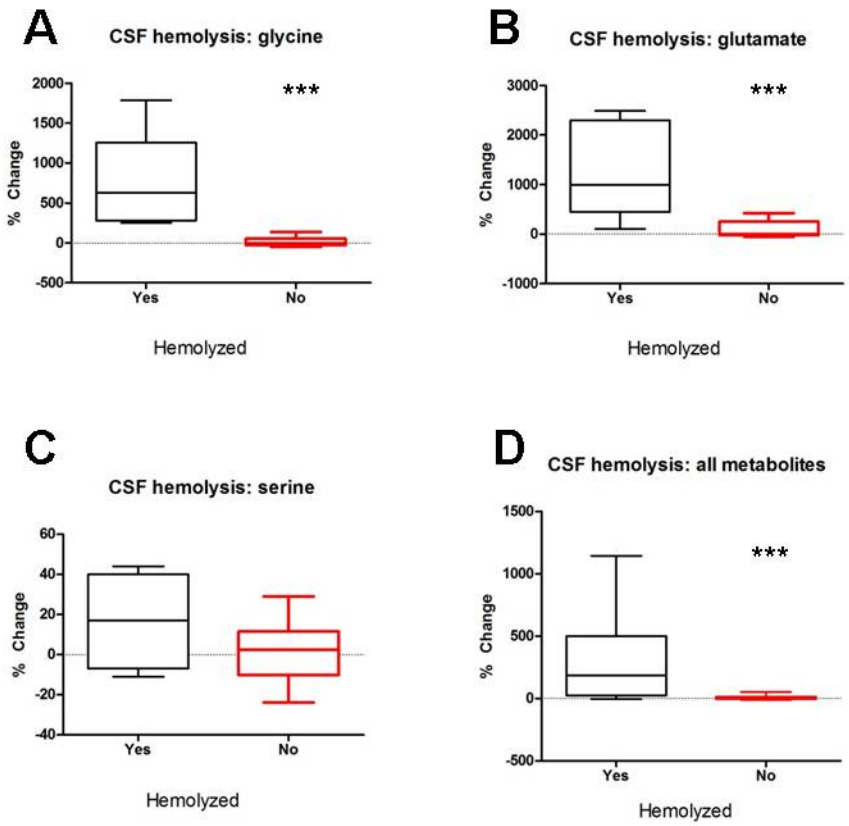
Supplemental Table 1. Physiological parameters during MCAO filament occlusion in adult male rats.

	SHAM	SMALL STROKE	LARGE STROKE
Number (N)	7	6	7
Stroke Volume (%)	--	9 ± 5	29 ± 2
Weight(gm)	374 ± 36	355 ± 51	346 ± 43
NPO (hrs)	17 ± 2	16 ± 4	17 ± 1
pH	7.33 ± 0.05	7.25 ± 0.10	7.36 ± 0.06
PCO2(mmHg)	57 ± 9	75 ± 17	52 ± 11
PO2(mmHg)	203 ± 17	207 ± 7	190 ± 21
Hct(%PCV)	37 ± 4	37 ± 4	37 ± 3
Hb(g/dL)	12.7 ± 1.2	12.6 ± 0.4	12.7 ± 0.9
Glucose(2h)	118 ± 14	141 ± 51	119 ± 42

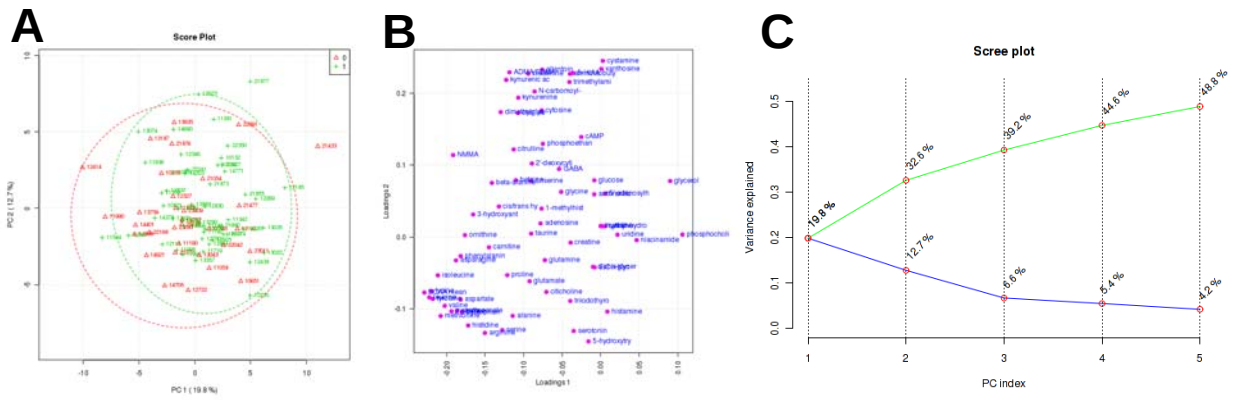
Supplemental Table 2. List of the metabolites measured by the metabolomics method. The mass spectrometry MRM transitions and the coefficient of variation (CV) in normal human plasma is provided for each metabolite.

Metabolite	Q1 mass	Q3 mass	CV in normal human plasma
1-methylhistamine	126.1	68.1	11.7%
2'-deoxycytidine	228.1	112.1	8.0%
3-hydroxyanthranilic acid	154	136.2	7.4%
3-OH Kynurenine	225	208	33.4%
5'-adenosylhomocysteine	385.1	136.3	13.0%
5-HIAA	192.3	146.2	7.0%
5-hydroxytryptophan	221.1	204	15.1%
adenosine	268.5	136.3	19.2%
ADMA/SDMA	203.1	70.3	4.1%
alanine	90	44	2.3%
allantoin	159	116	10.5%
alpha-glycerophosphocholine	258.3	104.1	26.8%
aminoisobutyric acid	104.1	86	4.1%
anthranilic acid	138	120	10.1%
arginine	175.1	70	5.8%
asparagine	133.1	74	3.1%
aspartate	134	74	6.5%
beta-alanine	90.01	72	3.6%
betaine	118.1	58	1.6%
cAMP	330.3	136.2	14.3%
carnitine	162.1	85	1.6%
carnosine	227.1	110	53.0%
choline	104.1	60	2.7%
cis/trans hydroxyproline	132.1	86.2	2.7%
citicholine	489.3	184.2	12.3%
citrulline	176	113.2	2.8%
creatine	132.1	90	2.7%
creatinine	114.1	44	2.6%
cystamine	153	108	5.1%
cytidine	244.2	112.2	53.4%
cytosine	112	95.1	12.1%
dimethylglycine	104.1	58	3.6%
GABA	104.1	87	13.0%
glucose	163.1	85	16.3%
glutamate	148.1	84	3.9%
glutamine	147.1	84	2.2%
glycerol	93	57	18.5%
glycine	76	48	4.4%
histamine	112	95	9.8%
histidine	156.1	110	6.4%
isoleucine	132.1	86.2	2.9%
kynurenic acid	190.2	144	10.9%

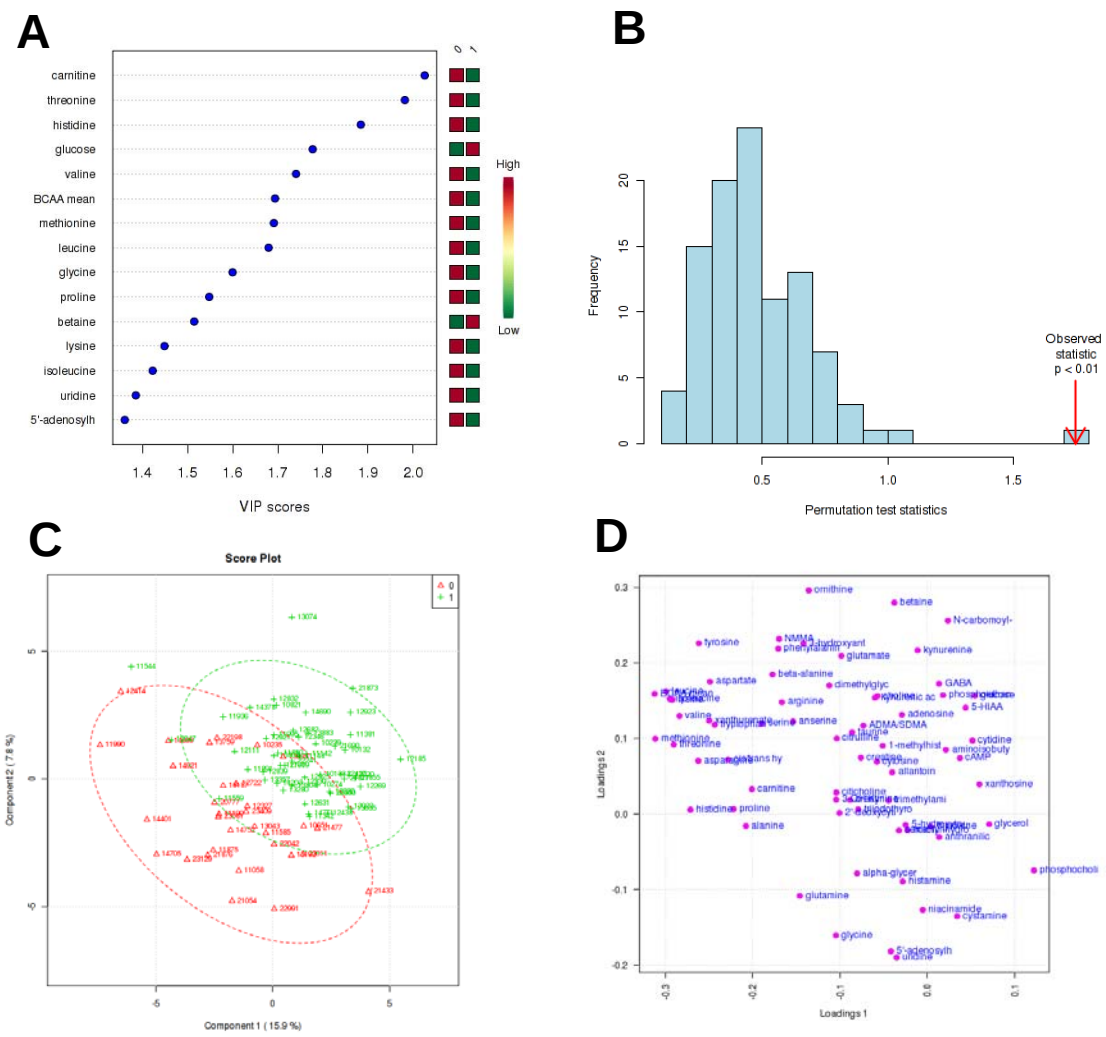
kynurenine	209	146	4.6%
leucine	132.1	86.2	2.4%
lysine	147.1	84	4.3%
methionine	150.1	61	4.1%
methyl-hydroxyisobutyric acid	119.1	87	35.2%
N-carbomoyl-beta-alanine	133.1	115	4.5%
niacinamide	123	80	23.2%
NMMA	189.1	70	6.1%
ornithine	133.4	70	7.3%
phenylalanine	166.1	120.2	3.6%
phosphocholine	184.5	125	5.0%
phosphoethanolamine	142	44	6.7%
proline	116.1	70	1.7%
serine	106	60	2.5%
serotonin	177.1	160	6.4%
taurine	126.2	44.1	3.1%
threonine	120.1	74	2.9%
thyroxine	777.8	732	4.6%
triiodothyronine	651.9	606.1	13.3%
trimethylamine-N-oxide	76.1	42	3.2%
tryptophan	205.5	188.3	3.7%
tyrosine	182.5	136.1	2.7%
uridine	245.2	113.1	13.7%
valine	118.1	72	1.8%
xanthosine	285.1	153	8.4%
xanthurenate	206	160	3.6%



Supplemental Figure 1. Effect of hemolysis on the concentration of metabolites in cerebrospinal fluid. Three representative metabolites are shown to demonstrate the range of effect of hemolysis. Glycine showed a 626% [373%, 1043%] accumulation, glutamate a 996% [493%, 1043%] increase and serine a 17% [0.5%, 29%] increase. The median increase in all metabolites in hemolyzed CSF was 285% [127%, 602%] compared to non-hemolyzed samples ($p<0.0001$). *** $p<0.001$.



Supplemental Figure 2. Principal component analysis of the acute plasma samples from subjects presenting to the emergency department with stroke symptoms. Panel A shows the score plot of the first two principal components, panel B shows loading plot of individual metabolites and panel C shows the scree plot of the PC model.



Supplemental Figure 3. Partial least square-discriminant analysis between control and stroke plasma samples reveals the variables with greatest impact in the variable importance in projection score (panel A). Permutation analysis confirms the significance of the model ($p<0.01$, panel B). The score plot of the first two components (panel C) and loadings plot of the individual metabolites are shown (panel D).

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