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Identification of Novel Biomarkers of Brain Damage in Patients with Hemorrhagic Stroke by Integrating Bioinformatics and Mass Spectrometry-Based Proteomics

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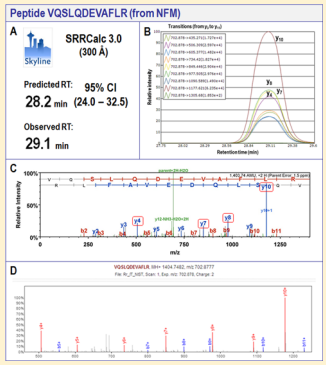
Supporting Information

ABSTRACT: Hemorrhagic stroke (HS) is a significant cause of mortality that requires rapid diagnosis and prompt medical attention. A time-efficient diagnostic test to assist in the early classification of patients with stroke would be of great value. The aims here were to (a) select "brain-specific" proteins using a bioinformatics approach, (b) develop selected reaction monitoring (SRM) assays for candidate proteins, and (c) quantify these proteins in cerebrospinal fluid (CSF). "The Human Protein Atlas" and the "Peptide Atlas" were used to select proteins specifically and abundantly expressed in brain tissue, excluding high-abundance plasma proteins. Protein extracts from brain tissue were used for SRM assay development of proteins of interest. The levels of 68 "brain-specific" proteins were measured by SRM in 36 age-matched patients, including individuals with HS (n = 15), ischemic stroke (n = 11), and controls (n = 10). Additionally, S100B was measured using an electrochemoluminometric immunoassay. CSF levels of S100B and eight of the "brain-specific" proteins (NSE, GFAP, α-Inx, MBP, MT3, NFM, β-Syn, and γ-Syn) were increased in a subset of samples from HS patients, especially in those individuals with intraventricular hemorrhage and poor outcome. Seven of these proteins (S100B, NSE, GFAP, α-Inx, MBP, NFM, and β-Syn) showed significant differences between patients with and without brain hemorrhage. Novel biomarkers of brain injury (α-Inx, NFM, and β-Syn) were identified in the CSF of patients with HS. Investigating the role of these proteins in blood with more sensitive methods is warranted.



KEYWORDS: *biomarker, cerebrospinal fluid, neurofilament, selected reaction monitoring, stroke*

INTRODUCTION

Stroke is a medical emergency caused by the interruption of blood supply to the brain. It is a significant cause of mortality and morbidity, with nearly 800,000 people in the United States suffering a new or recurrent stroke each year.[1] Every four minutes, someone dies of a stroke, rendering it the fourth leading cause of death in the United States after heart disease, cancer, and chronic lower respiratory diseases. Moreover, up to 30% of stroke survivors are left permanently disabled.[2]

Ischemic stroke (IS) represents 87% of all stroke cases, whereas hemorrhagic stroke (HS) accounts for 13% of cases (10% of intracerebral hemorrhages (ICH) and 3% of subarachnoid hemorrhages (SAH)).[1] Deterioration of stroke patients is common in the first few hours after symptom onset;[3−5] thus, rapid diagnosis and prompt medical attention is crucial. Moreover, differentiation between hemorrhagic and ischemic stroke is paramount to determine treatment options, such as intravenous thrombolytic therapy for IS[3] or hemostatic therapy for ICH.[4]

Noncontrast computed tomography (CT) scanning remains the cornerstone for diagnosis of stroke and is very sensitive for identifying acute hemorrhage.[4,5] However, it requires hospital admittance of patients, which causes a time-to-treatment delay[6] and exposure to potentially harmful ionizing radiation and is associated with high healthcare costs. CT scans are performed in a large number of patients with suspected SAH due to symptoms such as acute headache or syncope. However, more than 90% of cases have no indication of brain bleeding, but rather they are diagnosed with a benign headache or migraine.[7]

Therefore, a blood-based diagnostic test to assist in the early discrimination between HS and IS patients as well as to identify those patients that should be referred to a CT scan for a

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confirmatory diagnosis would be of significant value. Various proteins, such as S100 calcium-binding protein B (S100B), neuron-specific enolase (NSE)[8,9] and glial fibrillary acid protein (GFAP),[10] have been evaluated as diagnostic or prognostic biomarkers of brain damage in stroke patients. However, no protein biomarker has reached the clinic yet.

In the present study, we hypothesized that proteins abundantly and specifically expressed in the brain may be released and detected in the cerebrospinal fluid (CSF) of patients suffering a HS. Subsequently, these proteins may be detected in the circulation and be useful biomarkers for diagnosis and monitoring of stroke patients.

Our aims were: first, to identify "brain-specific" proteins using a bioinformatics approach; second, to develop selected reaction monitoring (SRM) assays for candidate protein biomarkers using brain tissue extracts; third, to quantify these proteins in CSF samples from patients with HS, IS, and control individuals using these mass spectrometry-based methods.

MATERIALS AND METHODS

Selection of Candidate Protein Biomarkers

The Human Protein Atlas (HPA),[11] version 10, was used to select proteins with high expression in brain tissue and low or absent expression in other body tissues (referred to as "brain-specific" proteins). The HPA provides protein expression profiles based on immunohistochemistry staining of up to 82 cell types from various human tissues (10 cell types from several human brain areas).

The normal tissue data from the 14 079 proteins included in this version of the HPA were downloaded from its Web site (http://www.proteinatlas.org/about/download) and reorganized by using an in-house Excel Macro (Microsoft Excel 2010). Then, proteins were ranked according to their staining intensity in brain cell types versus other body cell types [in-house scoring system: strong staining (score = 3), moderate (score = 2), weak (score = 1), or negative (score = 0)].

Proteins that were not previously identified by mass spectrometry in human brain tissue samples were excluded. Toward this goal, the proteome database named "Human Brain 2012-09" (including 19 samples and 2471 canonical proteins) from the Peptide Atlas[12] (http://www.peptideatlas.org/) and our own in-house brain proteome database (hippocampal tissue)[13] were consulted. Only "brain-specific" proteins identified in both databases with at least 10 observations (spectral counts) were selected.

To avoid high- or medium-abundance plasma proteins, the proteome database named "Human Plasma 2012-08" (including 151 samples and 3628 canonical proteins) from the Peptide Atlas was consulted to exclude proteins with more than 50 observations (with the exception of NSE).

Selected "brain-specific" proteins were contrasted with two proteomes of normal human CSF published by Pan et al.[14] and Schutzer et al.[15] in order to identify which proteins were not identified in the CSF of healthy individuals.

Selection of Unique Peptides for SRM Assay Development

Between 1 and 5 proteotypic peptides (length of 8 to 20 amino acids) per protein were initially selected. Peptide uniqueness was confirmed by searching against the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/).

Peptides previously identified in publicly available databases, such as the Global Proteome Machine (GPM) database[16] (http://gpmdb.thegpm.org/) and Peptide Atlas, identified in our in-house brain tissue proteome or used in the development of SRM assays by others[17] were preferred. Peptides with C-terminus cysteine and glutamine residues were excluded, and peptides with methionine residues were avoided, if possible.

Identification of Peptides in Brain Tissue Extracts

For SRM assay development, peptides were initially identified in a protein extract from a pool of three post-mortem frozen brain hippocampal tissues (control samples).[13]

Peptide identification was confirmed in four ways: First, retention times (RTs) were predicted using SRRCalc 3.0, 300 Å (Skyline software, version 1.4).[18] Second, the correlation of RTs between SRM and discovery gradients were examined. Third, the coelution of at least 6 transitions per peptide (from y_3 to y_{n-1}) were observed. Fourth, the observed fragmentation pattern of these peptides (SRM methods) was compared with the fragmentation pattern displayed in publicly available databases (Peptide Atlas and GPM database) and in our in-house brain tissue proteome (discovery data).

For RT prediction, a 0.2 mg/mL bovine serum albumin (BSA) solution with ten isotope-labeled standard peptides (SpikeTides TQL, JPT Peptide Technologies) was used. A multiplex SRM assay with 28 peptides (18 peptides from BSA and 10 isotope-labeled peptides) was run in a 60 min gradient and the measured RTs were utilized to predict the RT and 95% confidence intervals (CI) of target peptides, using Skyline software (SRRCalc 3.0).

Peptides were then organized in multiplex SRM assays with four peptides per method, according to their predicted time windows, trying to avoid or minimize overlap between scanning of peptides and thus increase the sensitivity of analysis. Transitions from y_3 to y_{n-1} were monitored for all peptides in aliquots of a brain tissue extract. For all those peptides positively identified and with information from our in-house brain proteome, the RT obtained in the discovery gradient (90 min) was compared with the RT obtained in the SRM gradient (60 min).

CSF Sampling

Thirty-nine samples from thirty-six age-matched patients were retrospectively selected from a CSF biobank at the Department of Clinical Biochemistry, Hospital Universitario Central de Asturias (HUCA), Spain; including samples from individuals with HS (n = 15), IS (n = 11), and controls (n = 10). Samples were stored at −80 °C until assayed. All patients admitted to the emergency department (ED) or intensive care unit (ICU) between January 2008 and December 2009 with CSF collection and diagnosis of stroke (except for "control" group) were eligible for inclusion. Exclusion criteria were age lower than 18 years, incomplete medical records, CSF collection after 10 days of symptoms onset, and blood contamination ("IS" and "control" groups). The final samples were selected in order to fulfill the calculated sample size from power analysis and to match the groups by age.

Ethics approval for sample collection was obtained from the institutional review board of HUCA, and the study was conducted according to the Declaration of Helsinki.

Patients admitted in the ED with symptoms (delirium, fever, headache) suspicious for meningitis but with a negative diagnosis for any neurological infectious disease or stroke were included in the "control" group. These individuals were diagnosed with benign headache, mild cognitive impairment, depression, and other non-neurological diseases.

Patients with a diagnosis of ischemic stroke after radiological confirmation with a CT scan, magnetic resonance imaging (MRI), or Doppler ultrasound and with CSF collection to rule out SAH (negative CT scan for blood) were included in the "IS" group.

Patients admitted to the ICU with a diagnosis of ICH or SAH and with external ventricular drainage (EVD) for management of elevated intracerebral pressure and hydrocephalus were included in the "HS" group. Two patients with diagnosis of SAH and collection of CSF in the first few hours after hospital admission were also included in this group.

CSF samples from "control" and "IS" groups were collected by lumbar puncture, whereas samples from patients with HS were collected from the EVD system, except in the two cases previously described where the sample was collected by lumbar puncture.

Clinical Variables

The following variables were recorded: age, sex, Glasgow Coma Scale (GCS) on admission, time span from hospital admission to sample collection, and results from brain imaging studies: CT scan (with and/or without contrast), MRI, and Doppler ultrasound.

Ischemic stroke ethiology was classified according to the TOAST criteria.[19] CT scans in patients with SAH were classified according to the Fisher scale.[20] Outcome assessment was based on the Glasgow outcome scale (GOS).

Analytical Procedures

CSF cell count, biochemical analysis (glucose and total protein concentration), and detection of xanthochromia were routinely performed and recorded before sample storage.

CSF S100B and hemolysis index were measured at the Biochemistry laboratory of HUCA, and then the samples were sent to the Lunenfeld−Tanenbaum Research Institute (Toronto) via FedEx in a container box (with dry ice). The concentration of S100B protein was measured using a fully automated electrochemoluminometric immunoassay in a Cobas 6000 system (Elecsys S100; Roche Diagnostics, Penzberg, Germany), with analytical range of 0.005−39 μg/L.

Reagents and Materials for SRM Analysis

RapiGest SF Surfactant was purchased from Waters Corporation (Milford, MA, USA). Dithiothreitol, iodoacetamide, proteomics-grade trypsin (T6567), and trifluoroacetic acid were from Sigma-Aldrich (Oakville, ON, Canada). Ammonium bicarbonate and acetonitrile (grade HPLC) were from Fisher Scientific (New Jersey, USA). Formic acid was from EMD (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q purification system (Millipore, Molsheim, France). Isotopically labeled standard peptides (SpikeTides L and TQL) were from JPT Peptide Technologies (Berlin, Germany). OMIX C18, 10 μL tips were from Agilent technologies (Lake Forest, CA, USA).

Sample Preparation

CSF samples were divided into at least 3 aliquots (100 μL) and stored at −80 °C until assayed.

Before SRM analysis, brain extracts and CSF samples were thawed at room temperature and centrifuged at 17000*g* for 10 min. Sample volumes were adjusted to 10 μg of total protein, and proteins were denatured and reduced with 0.05% RapiGest and 5 mM dithiothreitol, for 40 min at 60 °C. Alkylation was performed using 15 mM iodoacetamide for 60 min at room temperature in the dark. Next, dithiothreitol was added to a 10 mM final concentration (to quench excess iodoacetamide) and samples were incubated for 15 min at room temperature. Proteins were digested with trypsin for 24 h at 37 °C (trypsin to total protein ratio 1:20 (w/w)). After trypsinization, RapiGest was cleaved with 1% trifluoroacetic acid and the samples were centrifuged at 500*g* for 30 min for RapiGest precipitation. Peptides were purified and extracted using OMIX C18 tips and then eluted using 5 μL of 65% acetonitrile solution (0.1% formic acid). Finally, the peptides were diluted with 60 μL of water (0.1% formic acid). All steps were performed in a 96 well plate.

Liquid Chromatography−Mass spectrometry (LC−MS) conditions

Samples were loaded onto a 2 cm trap column (C18, 5 μm) with an inner diameter of 150 μm, and the peptides were eluted onto a resolving 5 cm analytical column (C18, 3 μm) with an inner diameter of 75 and 8 μm tip (New Objetive, Woburn, USA). The LC setup, EASY-nLC 1000 (Thermo Fisher, Odense, Denmark), was coupled online to a triple-quadrupole mass spectrometer (TSQ Vantage, Thermo Fisher, San Jose, USA) using a nanoelectrospray ionization source (nano-ESI, Thermo Fisher). A three-step 60/30 min gradient with an injection volume of 18 μL was used. Buffer A contained 0.1% formic acid in water, and buffer B contained 0.1% formic acid in acetonitrile. Peptides were analyzed by SRM assays with the following parameters: positive-ion mode, predicted collision energy values, optimized scan times, 0.2/0.7 Th of full width at half-maximum (fwhm) in Q1 and 0.7 Th in Q3, 1.5 mTorr Q2 pressure, tuned tube lens values and 1 V skimmer offset. In-silico digestion, fragmentation and prediction of collision energy were performed using Skyline software.

Selection of Transitions in SRM Assays for CSF Analysis

Three transitions per peptide were selected based on two main criteria: relative intensity (according to the results in brain tissue extracts) and presence of interferences. Transitions with the highest intensity were preferred. Presence of interferences was predicted by using the SRM collider software, version 1.4 (www.srmcollider.org).[21] SRM collider predicts unique ion signatures (UIS) for each peptide. The search parameters were: SSRCalc window, 10 arbitrary units; Q1 mass window, 0.2 Th; Q3 mass window, 0.7 Th; low and high mass threshold for transitions, 300 and 1500 Th, respectively; Genome, Human Peptide Atlas; consider isotopes up to 3 amu; one missed cleavage; find UIS up to order 3; and finally, charge check, modifications and all background ion series were selected.

Linearity and Limit of Quantification

A brain extract solution with 3610 fmol/μL of a mixture of isotope-labeled peptides (1000 fmoles of each peptide per injection) was initially prepared to study linearity. This solution was sequentially diluted (1:2) with brain tissue extract solution (0 fmoles of isotope-labeled peptides) to generate 13 points of calibration (1000, 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.9, 1.95, 0.98, 0.48, and 0.24 fmoles per injection). All these standard solutions were analyzed, in triplicate and from lowest to highest concentration. The limit of quantification (LOQ) was estimated as the lowest concentration within the linear range and with a coefficient of variation (CV) of 20%. The limit of detection was defined as the lowest concentration that could be detected with a signal-to-noise ratio of 3:1.

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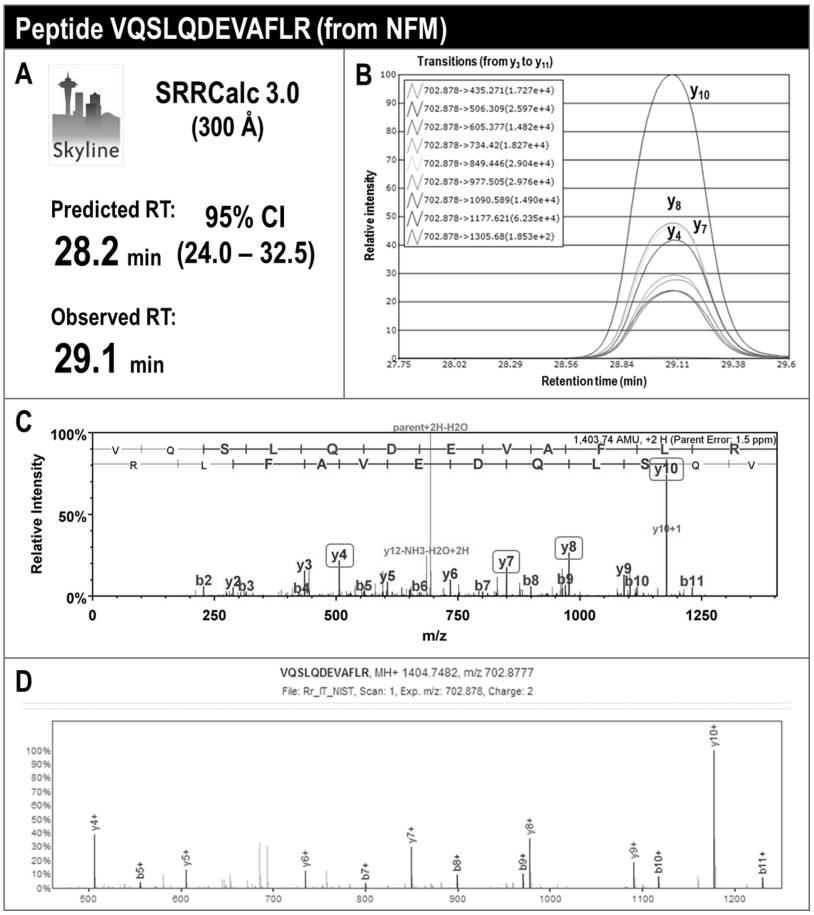

Figure 1. Predicted retention time (RT) and 95% confidence interval (CI) for peptide VQSLQDEVAFLR, according to SRRCalc. 3.0, and observed RT in a 60 min gradient (A). Coelution of y-ions (from y_3 to y_{11}) and relative intensity of transitions in parentheses, acquired in a TSQ Vantage (B). MS/MS spectrum of the doubly charged peptide VQSLQDEVAFLR (m/z = 702.8), acquired in an LTQ Orbitrap XL, and identification of b- and y-ions in Scaffold software (C). Consensus spectrum displayed in publicly available database "the Peptide Atlas" (D).

Data Analysis

CSF samples were analyzed in a stochastic order. The raw files were uploaded to Pinpoint software, version 1.0 (Thermo Fisher), which was used for quantification of areas under the curve (AUC).

For label free quantification, a known amount of two isotope-labeled peptides (one hydrophilic and one hydrophobic) was spiked into the CSF samples and the AUCs obtained were added and used for normalization of the AUC of endogenous peptides. The ratios $AUC_{light}/(AUC_{heavy1} + AUC_{heavy2})$ were adjusted by the CSF volume and used for comparison across samples.

For relative quantification, one isotope-labeled peptide (with the same peptide sequence) for each endogenous peptide was spiked into the CSF samples (same amount). Three transitions per peptide (one quantifier and two qualifiers) were monitored but only one transition (quantifier) was used for calculation of AUC. The ratios AUC_{light}/AUC_{heavy} were adjusted by the CSF volume and used for comparison across samples.

Statistical Analysis

Statistical analyses were performed with IBM SPSS Statistic software, version 20. A p-value <0.05 was considered statistically significant. Normal distribution was evaluated using Shapiro-Wilk test and by inspection of Q-Q plots. Means, medians, standard deviations, and interquartile ranges were calculated for descriptive and comparative purposes. Kruskal–Wallis and Mann–Whitney U (one-tailed) tests were performed for comparison between three and two groups of independent samples, respectively.

A power analysis for two-group independent sample t test was performed to determine the sample size. A minimum of 7 samples per group were required in order to have 80% power in detecting 3-fold increased protein concentration in the "HS" group compared to the "IS" group, at a significance level of 0.05, assuming a global CV lower or equal to 50%.

RESULTS

Selection of Candidate Protein Biomarkers

The top 390 proteins from the HPA v10, with the most intense expression in brain tissue and weakest expression in other body tissues, were initially selected. From these initial candidates, 228 proteins were not identified in either the brain Peptide Atlas proteome or in our in-house brain proteome. From the remaining 162 proteins, 53 were not identified in our in-house brain proteome (with more than 10 observations), 20 were not identified in the brain Peptide Atlas proteome (with more than 10 observations), and 13 proteins showed more than 50 observations in the plasma Peptide Atlas proteome (suggesting high abundance). Finally, 76 "brain-specific" proteins were selected for subsequent analyses (Supporting Information, Table S1). Thirty-five of these proteins were not found in the two normal CSF proteomes examined from the literature[14,15]

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(see also Supporting Information, Table S1), reflecting a very low or no secretion into the CSF under normal conditions.

Selection and Identification of Proteotypic Peptides

From the 76 candidate proteins, 205 unique peptides were initially selected.

The SRRCalc 3.0 was used to generate a linear regression (Supporting Information, Figure S1) between the RTs measured for 28 peptides (Supporting Information, Table S2) and their hydrophobicity indexes. The coefficient of correlation (R) obtained was 0.97, and the predicted time window for the 205 peptides was 8.5 min. Based on these time windows, 52 SRM assays with 4 peptides per method were prepared.

Out of the 76 initial candidates, 127 peptides from 68 proteins were identified in the brain tissue samples. The observed RTs were within the predicted time windows for 92% of the peptides (n = 117). Ten peptides were detected outside of the predicted time windows (Supporting Information, Table S3), probably because their RTs were very short or very long and, therefore, outside of the time range measured with the 28 peptides (from 8.9 to 39.4 min).

Out of 127 identified peptides, 114 were also found in our in-house brain proteome. The RTs observed in this discovery data set were compared to the RTs measured in the SRM assays, showing good correlation (Supporting Information, Figure S2).

The identification of peptides was based not only on the RT prediction but also on the observation of coelution of transitions and in the examination of fragmentation patterns. After observing the coelution of at least 6 transitions per peptide, the relative intensity of the top 4 transitions was compared with the intensity obtained in discovery data. The fragmentation patterns obtained in the MS/MS spectra from our in-house brain proteome were very similar to those displayed in publicly available databases (GPM and Peptide Atlas) for most of the identified peptides. For instance, the peptide VQSLQDEVAFLR from protein neurofilament medium polypeptide (NFM) had a predicted RT = 28.2 min (95% CI: 24.0−32.5), and the observed RT was 29.1 min. Nine transitions coeluted at this RT, being the most intense: $y_{10} \gg y_8 \sim y_7 \sim y_4$. The same transitions were found to be the most intense in both the MS/MS spectrum from our in-house brain proteome and the consensus spectrum from the Peptide Atlas (Figure 1). Other examples of peptide identification are displayed in Supporting Information, Figures S3 to S9.

When more than one peptide per protein was identified, the peptide with more intense transitions, with no amino acids prone to chemical modifications in its sequence, and with less overlap in the RT with other peptides was selected for subsequent analyses in CSF samples.

Clinical Characterization of Patients

The characteristics of the 36 patients included in the study are shown in Table 1. CSF samples from controls and IS patients had ≤6 white blood cells/mm^3 and negative xanthochromia.

Ischemic stroke cases (n = 11) were subclassified into large-arthery atherosclerosis (n = 2), cardioembolism (n = 3), small-vessel occlusions (n = 5), and undetermined etiology (n = 1) (Table 2).

Patients from the "HS" group (n = 15) were diagnosed with ICH (n = 7) or SAH (n = 8). Spontaneous ICH were located in basal ganglia (n = 5), thalamus (n = 1), and cerebellum (n = 1). All these patients showed secondary intraventricular hemorrhage (IVH). Five out of the eight patients with SAH had Fisher grade 4 scans, whereas the CT scan confirmed the absence of IVH in the other three patients (Fisher grades 1−3). The location of aneurysmal rupture was homogeneously distributed among the main brain arteries. Only one of these cases was a nonaneurysmal SAH (Table 2).

Table 1. Baseline Characteristics of the Study Population (n = 36)[a]

	study groups		
variable	HS	IS	control
patients (n)	15	11	10
mean age, years (SD)	53.9 (14.9)	60.5 (12.7)	56.1 (14.6)
women, n (%)	8 (53%)	4 (36%)	5 (50%)
CSF collection, days[b]	5 (0−9)	1 (0−3)	n/a
median CGS (interquartile range)	10 (4−13)[c]	14 (12−15)	n/a
Outcome (3 months), n (%)			
GOS 1	5 (33%)	4 (36%)	n/a
GOS 2	1 (7%)	0 (0%)	n/a
GOS 3	5 (33%)	1 (9%)	n/a
GOS 4	2 (13%)	2 (18%)	n/a
GOS 5	2 (13%)	3 (27%)	n/a
data missing, GOS > 1	0 (0%)	1 (9%)	n/a

[a]HS, hemorrhagic stroke; IS, ischemic stroke; SD, standard deviation; CSF, cerebrospinal fluid; GCS, Glasgow coma scale; GOS, Glasgow outcome scale; n/a, not applicable. [b]Expressed as median (minimum − maximum). [c]The score for one patient was missing.

Table 2. Ethiology of Ischemic Stroke Cases and Subarachnoid Hemorrhages[a]

	study group
ischemic stroke	
TOAST classification, n (%)	
large arthery atherosclerosis	2 (17%)
cardioembolism	3 (25%)
small vessel occlusion	5 (42%)
undetermined etiology	1 (8%)
hemorrhagic stroke	
variable	
subgroup, ICH (n)	7
subgroup, SAH (n)	8
Fisher CT grading scale, n (%)	
grade 1	1 (12%)
grade 2	1 (12%)
grade 3	1 (12%)
grade 4	5 (63%)
number of aneurysms, n (%)	
0	1 (12%)[b]
1	3 (38%)
2	2 (25%)
3	1 (12%)
unknown	1 (12%)[c]
aneurysm location, n (%)	
anterior communicating artery	2 (20%)
middle cerebral artery	2 (20%)
internal carotid artery	3 (30%)
posterior communicating artery	1 (10%)
posterior inferior cerebellar artery	2 (20%)

[a]ICH, intracerebral hemorrhage; SAH, subarachnoid hemorrhage. [b]Perimesencephalic nonaneurysmal subarachnoid hemorrhage. [c]Massive subarachnoid hemorrhage with patient death.

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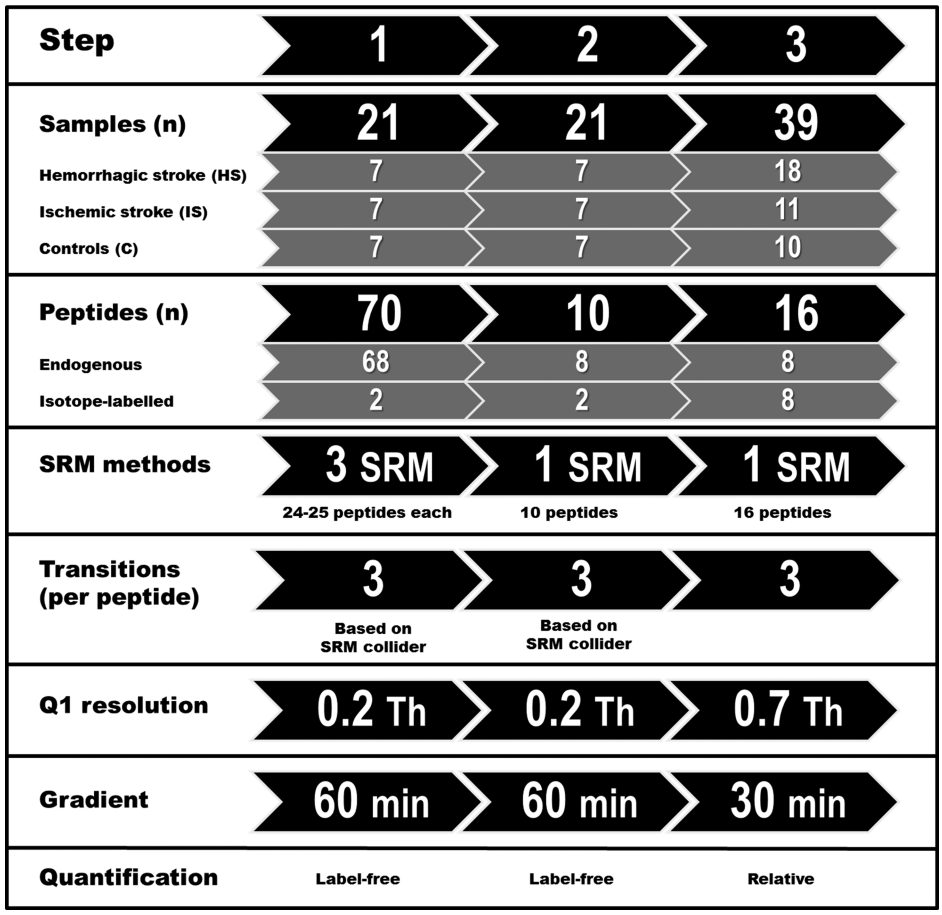

Figure 2. Analysis of CSF samples in a multistep process. For details, see text.

Table 3. Analytical Range and Limit of Quantification for 8 Endogenous Peptides from 8 Protein Biomarkers[a]

protein	peptide	transitions (quantifier)	LOQ	CV	analytical range	R^2
NSE	IEEELGDEAR	580.7−918.4	0.24	4%	0.24−1000	0.9999
		585.7−928.4			0.24−3.9	0.9997
GFAP	DNLAQDLATVR	608.3−873.4	0.24	5%	0.24−1000	0.9999
		613.3−883.4			0.24−3.9	0.9999
α-Inx	ALEAELAALR	528.8−872.4	0.49	9%	0.49−1000	0.9999
		533.8−882.4			0.49−7.8	0.9987
MBP	GVDAQGTLSK	488.2−819.4	0.24	12%	0.24−1000	0.9999
		492.2−827.4			0.24−3.9	0.9996
MT3	GGEAAEAEAEK	531.2−747.3	0.98	6%	0.98−1000	0.9998
		535.2−755.3			0.98−15.3	0.9996
NFM	VQSLQDEVAFLR	702.8−1177.6	0.49	13%	0.49−1000	0.9998
		707.8−1187.6			0.49−7.8	0.9992
β-Syn	EGVVQGVASVAEK	636.8−760.4	0.24	10%	0.24−1000	0.9999
		640.8−768.4			0.24−3.9	0.9999
γ-Syn	TVEEAENIAVTSGVVR	837.4−788.4	1.95	7%	1.9−1000	0.9997
		842.4−798.4			1.9−31.2	0.9971

[a]LOQ, limit of quantification (fmoles per injection); CV, coefficient of variation (triplicates) at LOQ; R^2, coefficient of determination

Analysis of CSF Samples

For CSF analysis, a multistep process was followed (Figure 2). In the first step, 21 CSF samples (7 from HS patients, 7 from IS patients, and 7 from controls) were analyzed. Sixty-eight endogenous peptides (one per protein) were monitored in these samples. Three multiplex SRM assays (60 min gradient) with 24−25 endogenous and two isotope-labeled peptides were prepared. Three transitions per peptide were selected based on the SRM collider (Supporting Information, Table S4). After this first analysis, 32 peptides were not detected in any of the CSF samples and 28 peptides were detected in some of the CSF samples, but the assay sensitivity was very poor or the peptides were not clearly elevated in samples from patients with HS. Only 8 peptides from proteins NSE, GFAP, α-internexin (α-Inx), myelin basic protein (MBP), metallothionein III

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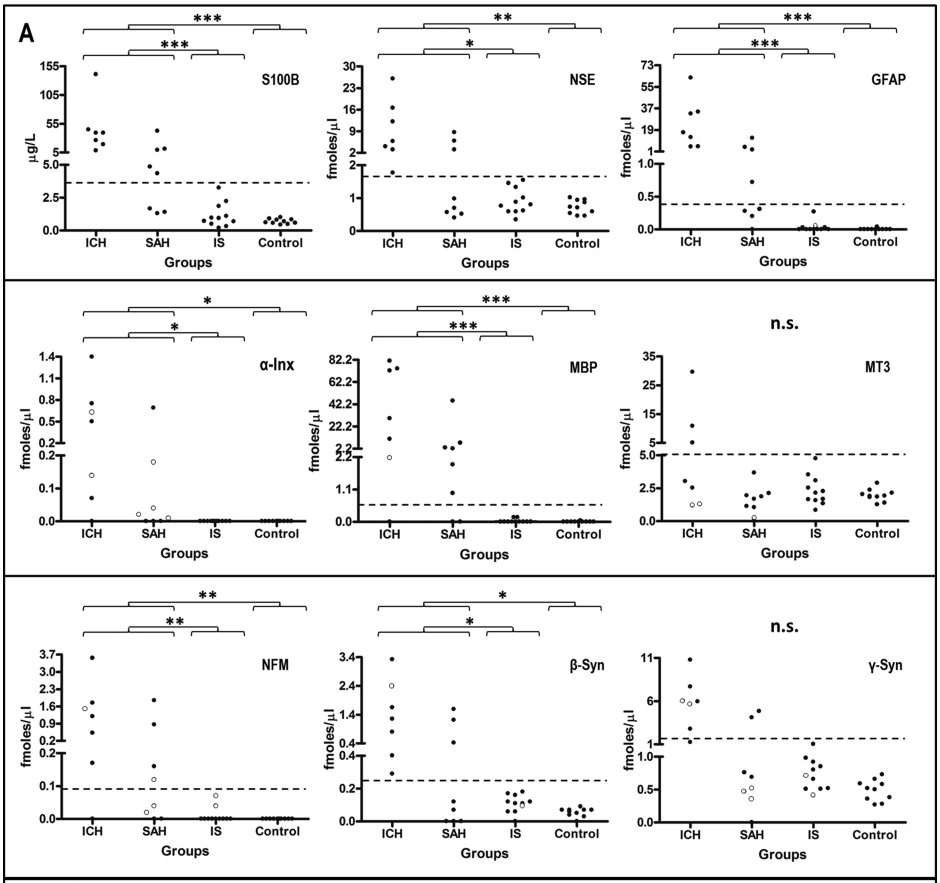

B

Marker	ICH versus IS	SAH versus IS	HS versus IS
S100B	100 (100)	63 (63)	80 (80)
NSE	100 (100)	38 (38)	67 (67)
GFAP	100 (100)	50 (50)	73 (73)
α-Inx	57 (86)	13 (63)	33 (73)
MBP	71 (86)	75 (75)	73 (80)
NFM	71 (86)	38 (50)	53 (67)
β-Syn	86 (100)	38 (38)	60 (67)

Figure 3. Results for S100B and eight other protein biomarkers (SRM assay) in CSF samples (n = 36). The groups ICH, SAH, IS, and Control are shown. The filled (●) and open circles (○) represent the values above and below the LOQ, respectively. The dashed lines define the biomarker concentration cutoff that discriminates patients with HS from patients with IS (at 100% specificity). For α-Inx, the cutoff was close to zero. Values below the LOQ were considered as zero in the statistical analysis. P value: $p < 0.001$ (***); $p < 0.01$ (**); $p < 0.05$ (*); n.s., not significant (A). The clinical performance (sensitivity (in percentage) at 100% specificity) of markers with significant differences is summarized in the table, including only values above the LOQ and all values (between parentheses) (B).

(MT3), NFM, β-synuclein (β-Syn), and γ-synuclein (γ-Syn) were selected for further analysis.

In the second step, a new aliquot of these 21 samples was analyzed with a multiplex SRM assay (60 min gradient) containing these 8 endogenous peptides and two isotope-labeled peptides. The results observed in the first analysis were confirmed in this second analysis.

In the third step, the 39 CSF samples collected as previously described and one pool of plasma samples were analyzed with a multiplex SRM assay (30 min gradient) with 8 endogenous peptides and 8 isotope-labeled peptides (Supporting Information, Table S5 and Figure S10). The plasma pool was used to check for interferences. None of the endogenous peptides were detected in the plasma sample.

Calibration Curves and Limit of Quantification

The SRM assays showed good linearity (coefficient of determination, $R^2 > 0.99$) in both the entire analytical range, from 1000 fmoles to the LOQ, as well as in the lower range (five lower concentration standards) (Table 3 and Supporting Information, Figures S11 to S18). The transition (out of three) with the highest relative intensity and without interferences, for both the endogenous and the isotope-labeled peptides, was used for peptide quantification.

The LOQs (per injection) were 0.24 fmoles for NSE (CV = 4%), GFAP (CV = 5%), MBP (CV = 12%), and β-Syn (CV = 10%), 0.49 fmoles for α-Inx (CV = 9%) and NFM (CV = 13%), 0.98 fmoles for MT3 (CV = 6%), and 1.95 fmoles for γ-Syn (CV = 7%).

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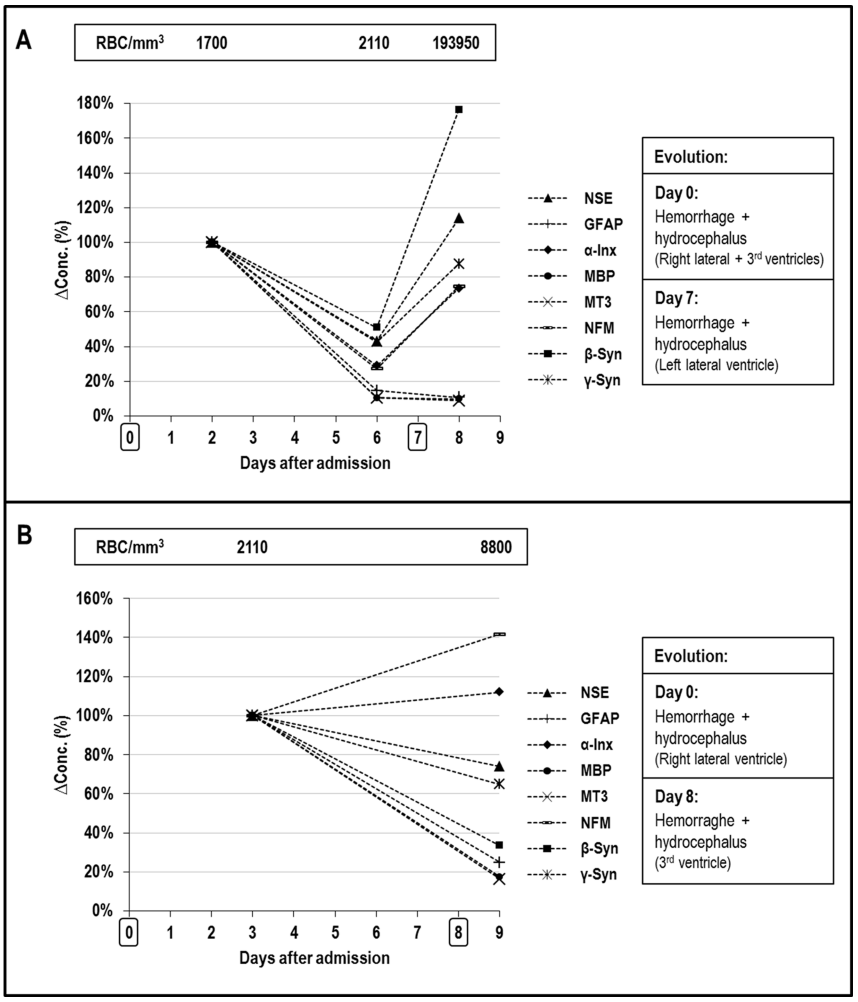

Figure 4. Dynamic changes of protein concentration (expressed as variation of concentration (ΔConc.) with respect to the first sample) in CSF samples from two patients with ICH (A and B). RBC = red blood cells.

CSF Concentration of Protein Biomarkers

S100B was significantly elevated ($p < 0.001$) in the HS group, especially in the CSF samples from patients with ICH and some of the patients with SAH (Figure 3 and Supporting Information, Table S6). The other eight proteins quantified with the multiplex SRM assay were also elevated in some of the CSFs from HS patients, with higher concentrations in the samples from patients with ICH.

Proteins GFAP and MBP showed the greatest differences ($p < 0.001$) between patients with HS and IS. GFAP was detected in 18 CSF samples and quantified in 17 of them. The concentration of this protein was increased in all ICH samples and in four CSFs from patients with SAH and IVH (Fisher CT scan grade 4). MBP was detected in 15 CSFs and quantified in 14 of them. Its concentration was elevated in 5 out of 7 patients with ICH and in patients with SAH and Fisher grades 3−4 ($n = 6$).

The CSF concentration of proteins NFM ($p < 0.01$), NSE, α-Inx, and β-Syn ($p < 0.05$) was also significantly increased in the HS group. NFM was detected in 14 CSFs and quantified in 8, all of them from patients with HS and IVH. NSE was elevated in all ICH patients and in three patients with SAH and Fisher grade 4. Protein α-Inx was detected in 11 CSF samples (6 ICH and 5 patients with SAH and Fisher grade 3−4) but quantified in only 5 of them. Protein β-Syn was detected in 31 samples but with a concentration below the LOQ in 2 of them. This protein was elevated in 6 patients with ICH and in 3 patients with SAH and IVH.

The CSF concentration of proteins MT3 and γ-Syn did not significantly differ among groups. Protein γ-Syn was detected in 35 CSFs and quantified in 28 of them. This protein was elevated in 4 samples from patients with ICH and 2 samples from patients with SAH. MT3 was detected in all CSFs but with a concentration below the LOQ in 3 of them. It was increased in only 3 samples from patients with ICH.

Finally, when considering the concentrations (including the results below the LOQ) in the 12 CSF samples from patients with HS and IVH and compared to the concentration in the IS and control samples (Supporting Information, Table S6), proteins S100B, GFAP, α-Inx, and MBP were elevated in 11 of these samples, and proteins NSE, NFM, and β-Syn were elevated in 10. However, proteins γ-Syn and MT3 were only elevated in 8 and 3 CSF samples, respectively.

Monitoring Protein Concentration

Five CSF samples from two patients with ICH and collected at different time points after hospital admission were assayed in order to monitor protein concentrations.

In the first patient, three CSF samples were collected 2, 6, and 8 days after admission. This patient had IVH in the right lateral and third ventricles and hydrocephalus. In a new CT

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scan 7 days after admission, an abundant hemorrhage in the left lateral ventricle and hydrocephalus were observed. A 90-fold increase in red blood cell counts was observed on a CSF sample at day 8 compared to day 6 (Figure 4A).

In the second patient, two CSF samples were available (collected 3 and 9 days after admission). This patient had IVH in the right lateral ventricle and hydrocephalus. Eight days after admission, a mild hemorrhage in the third ventricle and accumulation of fluid were observed. A 4-fold increase in red blood cell count was found on a CSF sample at day 9 compared to that of day 3 (Figure 4B).

In both patients, the dynamic changes in the levels of proteins GFAP, MT3, and MBP were very similar, with a clear lowering in the CSF concentration from the first to the last sample. On the other hand, proteins α-Inx and NFM changed by a similar percentage but in the opposite direction because the CSF concentration of both proteins was raised in the samples with increased bleeding.

The concentration of proteins NSE, β-Syn, and γ-Syn evolved differently in these two patients because in the first patient these proteins were increased between the second and third samples and in the second patient were decreased, probably due to the differences in the collection time between samples (2 and 5 days, respectively) and bleeding intensity.

DISCUSSION

The identification of biomarkers for stroke and traumatic brain injury (TBI) is a very active area of research. To date, various biomarkers of stroke have been studied; however, none of them have reached the clinical setting, in large part due to the lack of sufficient sensitivity and specificity.

Given the complexity and heterogeneity of stroke etiology, it is unlikely that a single biomarker can be successfully used in these patients. Therefore, it has been suggested that integrated panels of biomarkers with specific and complementary characteristics may assist in the diagnosis, risk assessment, treatment selection, and prediction of clinical outcomes of patients with brain damage. These could be biomarkers of distinct pathobiological processes, such as necrosis, axonal injury, apoptosis, demyelination, microgliosis, and neuroregeneration.[22]

The goal of this study was to identify novel proteins that can be used as biomarkers of brain damage and hemorrhage in stroke patients. In particular, we focused on biomarkers of cell death (glial, neuronal, and endothelial cells) because necrosis, apoptosis, and autophagy cell death pathways are activated early after hemorrhage.[23]

The candidate proteins selection was based on the hypothesis that proteins specifically expressed in brain cells would be valuable biomarkers in the case that their concentration in CSF correlates with the extension of brain damage. Detectable or increased levels of these proteins in the bloodstream will presumably result from the disruption of the blood−brain barrier.

The HPA provides a wide protein expression profile that enables the identification of tissue-specific proteins. Proteins not identified in brain proteomes were excluded because it may mean that these proteins do not have peptides that can be detected by mass spectrometry (technical limitation) or that they are low-abundance brain proteins and, therefore, their detection in proximal fluids would be challenging. Proteins with relatively high plasma concentration were also discarded to avoid the risk that elevation of their levels in the CSF samples from patients with HS was due to blood contamination and not because they were released from the injured brain.

The already known neuromarkers S100B and GFAP fulfilled our criteria for biomarker selection, validating the potential value of the other candidate biomarkers identified in this study. NSE (also a known neuromarker) did not fulfill all criteria (>1000 spectral counts in the plasma Peptide Atlas proteome), but it was included as a positive control.

CSF samples from patients with HS and IS were analyzed to identify potential biomarkers, able to differentiate these two types of stroke. Samples from control individuals were utilized to establish the baseline CSF concentrations of these proteins. In order to have a first piece of information about the dynamics of release and clearance of proteins from the CSF, five samples from two of the patients with ICH that were collected at different time points were also analyzed.

The outcome of HS patients was associated with the severity of the bleeding in the initial CT scan. Patients with IVH (n = 12) had a worse outcome, GOS 1−3 (except in one case with GOS 4), than patients without IVH (n = 3), GOS 4−5. It is known that the IVH is an independent predictor of poor outcome, probably due to the induction of hydrocephalus and inflammatory response.[24]

Nine proteins were analyzed in the entire set of CSF samples (n = 39) after showing increased levels in a subset of samples from HS patients. Seven of these proteins (S100B, NSE, GFAP, α-Inx, MBP, NFM, and β-Syn) showed significantly higher CSF levels in patients with HS than in patients with IS and controls; the highest concentrations were found in patients with IVH and poor outcome. This is probably due to the destruction of brain tissue and cell death. Because more than one cell death pathway is active after HS,[23] the release of these proteins (expressed by different brain cell types and structures) into the extracellular fluid could provide complementary information about the physical disruption to the brain's cellular architecture and, therefore, about the extension of the brain damage.

Although S100B and GFAP are expressed primarily by astrocytes, NSE is a glycolytic pathway enzyme considered an indicator of neuronal damage. MBP is a protein involved in the maintenance of the structural integrity of the myelin sheath in oligodendrocytes and Schwann cells, which enables rapid transmission of nerve impulses through axons.[25] β-Syn is mainly located in the presynaptic terminals of neurons, and NFM is one of the neurofilament triplet proteins (neurofilament light, medium and heavy polypeptides; NFL, NFM and NFH) which, together with α-Inx, form the four major protein components of the neuronal intermediate filaments.[26] These three proteins (β-Syn, NFM, and α-Inx) are probably released into the CSF after disintegration of the axonal membrane, being potential indicators of neuronal death and axonal loss.

S100B and NSE are the two most widely investigated neuromarkers. Its concentration is increased in the CSF and blood from patients with SAH and ICH[9,27−30] and is associated with poor outcome[9,28,30] and detection of cerebral infraction and intracranial hypertension.[9] The concentration of NSE protein is also elevated in the CSF and serum from patients with SAH[9] and ICH[29] and is associated with poor outcome.

The elevation of MBP in the CSF of patients with SAH was described by Hirashima et al.,[31] suggesting that it may be an indicator of severity of brain damage due to vasospasm. MBP concentration is also increased in the CSF of children with TBI[32] and in the serum of patients with ICH.[33]

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GFAP was shown to be elevated in patients with SAH both in the CSF, with a slower clearance in patients with poor outcome,[34] and serum, with increased levels in patients with secondary events (rebleeding or ischemia).[35] Serum GFAP concentration is also elevated in patients with ICH and can be used to differentiate IS and ICH.[10,36,37]

To our knowledge, this is the first study showing increased levels of proteins NFM and α-Inx in the CSF from patients with HS. The other two proteins of the neurofilament triplets, NFH and NFL, were found to be elevated in patients with HS. NFH was increased in the CSF of patients with SAH and ICH,[38−40] and it has been described as a biomarker of axonal degeneration and adverse outcome. Zanier et al.[41] studied NFL in the CSF from patients with SAH. NFL levels were increased, with higher concentrations in individuals with early cerebral ischemia. However, NFL concentrations were not associated with secondary events.

These two proteins (NFL and NFH) were not included in the present study, because NFL has not been studied as part of the HPA and, therefore, is not included in the database. NFH was included in the initial list of 390 candidates but then discarded because of the high number of spectral counts in the plasma Peptide Atlas proteome (723 observations).

According to the information extracted from the HPA and Peptide Atlas, NFM and α-Inx seem to be more specific and abundant brain-proteins and, therefore, potentially better neuromarkers than NFH. Although NFH has a positive staining in 21 cell types (six from brain), NFM and α-Inx have a positive staining in only 7 and 8 cell types, respectively (4 from brain). Moreover, NFM and α-Inx seem to be more abundant in brain and less abundant in plasma than NFH. The ratio in the number of observations between the brain and plasma Peptide Atlas proteomes for protein NFH (162/723) is substantially lower than the same ratio for proteins NFM (942/16) and α-Inx (508/16).

Proteins β- and γ-Syn are two of the three members of the family (together with α-synuclein). These proteins have regulatory and modulatory functions in the central nervous system, including regulation of synaptic functions and vesicle traffic, plasticity, and neurotransmitter release.[42] Protein β-Syn inhibits α-synuclein aggregation and fibril formation, and it has been associated with neurodegenerative diseases.[42] On the other hand, protein γ-Syn has been associated with neurodegenerative diseases and cancer.[42,43] However, these two proteins have not been studied as stroke biomarkers.

Finally, MT3 is one member of the metallothionein family, which includes three cysteine-rich proteins (MT1, MT2, and MT3). MT3 is synthesized predominantly by astrocytes within the brain and is actively secreted in normal conditions. Its synthesis is up-regulated by reactive astrocytes after a brain injury.[44] However, little is known about the usefulness of this protein as a stroke biomarker.

With regard to the dynamics of protein concentration after the initial brain insult, a homogeneous pattern was not found. Although some proteins (GFAP, MBP, and MT3) had a peak of concentration after the initial episode and then dropped, other proteins (α-Inx, NSE, NFM, β-Syn, and γ-Syn) showed a rebound in their CSF concentration after a secondary event (rebleeding and hydrocephalus). Only proteins NFM and α-Inx showed an elevation in the CSF concentration of two samples (two patients) collected after this secondary event (Figure 4). Further analyses are needed to fully understand the release and clearance of these biomarkers.

Mass spectrometry has emerged as a technology that can overcome some of the limitations of traditional immunoassays. SRM assays require shorter times for method development and have multiplexing capabilities.[45] These advantages make this technology a good alternative for biomarker discovery.[46,47] However, SRM methodology also has some limitations that need to be considered.

The initial peptide identification used in this study for SRM assay development was performed without isotope-labeled standard peptides. Four different approaches were carried out to obtain a reliable identification of peptides. However, peptide identity cannot be ensured unless heavy-isotope labeled peptides are included in the analysis. To overcome this limitation, the selection of transitions for label-free quantification of peptides was based on the SRM collider, software that allows the identification of UIS of target peptides, thus avoiding or minimizing the misidentification of analytes.

Furthermore, it was not possible to develop SRM assays (no appropriate peptides found) for 8 of the 76 initial candidates (including S100B). This protein was analyzed by immunoassay and used as a positive control.

Another important limitation of SRM in this study was that assay sensitivity depends on sample complexity (total protein concentration). CSF samples from patients with abundant hemorrhage had an increased total protein concentration due, in large part, to the blood contamination. Therefore, the sensitivity in these samples was lower because at higher TP concentration, the CSF volume assayed was lower. This was especially evident in two of the CSF samples from patients with ICH (patients 6 and 7, Supporting Information, Table S6), where the total protein concentration was >10 g/L and the CSF volume assayed was around 1 μL. In these two samples, some of the proteins (α-Inx, MBP, MT3, NFM, β-Syn, and γ-Syn) were not identified or were identified but with concentrations below the LOQ.

Finally, there are a few aspects related to the biological fluid selected for our biomarker discovery study that need to be considered. CSF is a biological fluid with great potential to reveal brain-specific biomarkers (about 20% of the CSF proteins are derived from brain[48]), although it has limited clinical applicability. The proteomic analysis of blood is challenging because, due to its complexity, only medium- to high-abundance proteins can be detected. However, brain-derived proteins are usually present at low concentrations in the CSF (at the low ng/mL level) and even lower concentrations in the bloodstream.

On the other hand, two different procedures (EVD and lumbar puncture) were used for CSF collection from patients of "HS" and "IS" groups, respectively. Because a negative craniocaudal concentration gradient for brain-derived proteins has been suggested,[49] the extraction procedure could constitute a drawback for interpreting the results. However, Brandner et al.[50] have recently reported that there are no differences in the concentration of brain-derived proteins S100B and NSE between the ventricular and lumbar CSF compartments. Another limitation is the different timing of CSF sample collection (days after symptom onset) between groups. However, we believe that the detection of significantly increased concentrations of well-known stroke biomarkers (S100B, NSE, and GFAP), little-known proteins (MBP), and novel proteins (NFM, α-Inx and β-Syn) in the CSF samples of patients with HS serves as a proof of concept and validates the approach used.

CONCLUSION

Novel biomarkers of brain injury (NFM, α-Inx and β-Syn) were identified in the CSF of patients with HS. More sensitive assays (such as immuno-SRM or ELISA) need to be developed in order to perform validation studies, both in CSF and blood, to elucidate the clinical relevance of these proteins as biomarkers of stroke.

ASSOCIATED CONTENT

Supporting Information

Calibration curves and additional information on retention times and SRM assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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NONSTANDARD ABBREVIATIONS

α-Inx, α-internexin; AUC, area under the curve; β-Syn, β-synuclein; BSA, bovine serum albumin; CI, confidence interval; CSF, cerebrospinal fluid; CT, computed tomography; CV, coefficient of variation; ED, emergency department; EVD, external ventricular drainage; γ-Syn, γ-synuclein; GCS, Glasgow coma scale; GFAP, glial fibrillary acidic protein; GOS, Glasgow outcome scale; HPA, Human Protein Atlas; HS, hemorrhagic stroke; HUCA, Hospital Universitario Central de Asturias; ICH, intracerebral hemorrhage; ICU, intensive care unit; IS, ischemic stroke; IVH, intraventricular hemorrhage; LC, liquid chromatography; LOQ, limit of quantification; MBP, myelin basic protein; MRI, magnetic resonance imaging; MS, mass spectrometry; MT3, metallothionein III; NFL, neurofilament light polypeptide; NFM, neurofilament medium polypeptide; NFH, neurofilament heavy polypeptide; NSE, neuron-specific enolase; R, coefficient of correlation; R^2, coefficient of determination; RT, retention time; SAH, subarachnoid hemorrhage; S100B, S100 calcium-binding protein B; SRM, selected reaction monitoring; TBI, traumatic brain injury; UIS, unique ion signature

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