ARTICLE CLASS OF EVIDENCE

Blood Biomarkers to Differentiate Ischemic and Hemorrhagic Strokes

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Abstract

Objective

To validate a panel of blood biomarkers to differentiate between ischemic stroke (IS) and intracerebral hemorrhage (ICH) in patients with suspected stroke.

Methods

Patients with suspected stroke admitted within 4.5 hours after onset were enrolled. Blood samples were collected at hospital admission. Glial fibrillary acid protein (GFAP), retinol binding protein 4 (RBP-4), N-terminal proB-type natriuretic peptide (NT-proBNP), and endostatin were measured by immunoassays. Cutoff points were obtained for 100% specificity for IS. A high-sensitivity assay to measure GFAP and rapid point-of-care tests (POCTs) to measure RBP-4 and NT-proBNP were used in subsets of patients. Biomarker panels were evaluated in another cohort of 62 stroke mimics.

Results

A total of 189 patients (154 IS and 35 ICH) were enrolled. Patients with IS had higher RBP-4, NT-proBNP, and endostatin and lower GFAP levels than patients with ICH. The best biomarker combination for the identification of IS was RBP-4+NT-proBNP, which was able to identify 29.7% of patients with IS with 100% specificity. In the subset of patients for whom GFAP was measured with the high-sensitivity assay, RBP-4, NT-proBNP, and GFAP identified 51.5% of patients with IS with 100% specificity. When stroke mimics were included, specificities were reduced to 98.4 and 96.8%, respectively. POCTs of RBP-4 and NT-proBNP showed results similar results to those of conventional ELISAs.

Conclusions

A biomarker panel including RBP-4, NT-proBNP, and GFAP provided moderate but potentially useful sensitivity rates at 100% specificity for IS diagnosis. If confirmed in future studies, this strategy might allow prehospital treatment in selected patients.

Classification of Evidence

This study provides Class I evidence that a biomarker panel including RBP-4, NT-proBNP, and GFAP distinguishes IS from ICH with moderate accuracy.

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Glossary

AF = atrial fibrillation; **CV** = coefficient of variation; **GFAP** = glial fibrillary acid protein; **ICH** = intracerebral hemorrhage; **IS** = ischemic stroke; **mRS** = modified Rankin Scale; **MT** = mechanical thrombectomy; **NIHSS** = NIH Stroke Scale; **NT-proBNP** = N-terminal proB-type natriuretic peptide; **OD** = optical density; **OR** = odds ratio; **POC** = point-of-care; **POCT** = point-of-care test; **RBP-4** = retinol binding protein-4; **TIA** = transient ischemic attack; **tPA** = tissue plasminogen activator.

Stroke represents one of the main causes of morbidity and mortality worldwide. Recent data from the American Heart Association, specific to the United States, indicate that stroke is ranked fifth among all causes of death. In 2016, stroke accounted for 1 of every 19 deaths. On average, every 40 seconds, someone in the United States has a stroke, and every 3 minutes, someone dies of a stroke[1]; worldwide, stroke ranks even higher in mortality.

Currently, IV thrombolysis with recombinant tissue plasminogen activator (tPA) and mechanical thrombectomy (MT) represent the only acute-phase therapies that have been demonstrated to improve clinical outcome in ischemic stroke (IS).[2,3] Both therapeutic approaches aim to recanalize the occluded vessel, thereby restoring blood flow to the ischemic area. However, for both therapies, the time from stroke onset to recanalization achievement remains a crucial factor.[4,5] In fact, for IV tPA, the number of patients needed to treat to avoid a case of functional disability increases from only 4.5 within the first 90 minutes to 14 when the drug is administered from 3 to 4.5 hours.[6] In the case of intracerebral hemorrhage (ICH), no acute treatment has demonstrated efficacy, although intensive blood pressure lowering and the administration of inhibitors of coagulation factors are under evaluation.[7]

Prehospital differentiation between IS and ICH would allow early initiation of IV thrombolysis. The administration of tPA at the prehospital level in mobile stroke units has demonstrated feasibility and efficacy, as well as a reduced time from symptom onset to the initiation of tPA.[8,9] However, portable CT scans are required to accurately rule out ICH. Due to these technical and financial limitations, mobile stroke units are scarce resources and are more commonly used for research purposes than for clinical applications.

An alternative approach to prehospital stroke care might be represented by the use of blood biomarkers to differentiate between IS and ICH. A large amount of evidence supports the role of the glial marker glial fibrillary acid protein (GFAP) in the differentiation of IS from ICH.[10,11] A few years ago, we identified the biomarker retinol binding protein-4 (RBP-4) as a marker of IS and, when combined with GFAP, it provided high specificity, but low sensitivity, for differentiation of the subtypes.[12] In addition, in the Stroke-Chip study, N-terminal proB-type natriuretic peptide (NT-proBNP) and endostatin were able to provide 80% accuracy in the differentiation of IS and ICH when combined with clinical variables.[13]

In this study, we aimed to validate and develop a panel of blood biomarkers with enough accuracy to guide prehospital thrombolysis in selected patients with IS. In addition, we set up point-of-care (POC) devices for these biomarkers with lateral-flow immunoassays to allow a fast and reliable measure that could be performed outside hospital facilities.

Methods

Participants

From December 2013 to July 2015, patients with suspected stroke within 4.5 hours after symptom onset were consecutively enrolled at hospital admission in the Emergency Department of Vall d'Hebron University Hospital. Stroke diagnosis was performed according to WHO criteria[14] and confirmed by neuroimaging, which consisted of CT, CT angiography, and CT perfusion in most cases. At hospital admission, a complete medical history regarding vascular risk factors and medications was obtained. Stroke severity was assessed at admission with the NIH Stroke Scale (NIHSS). To focus on potentially eligible patients for IV tPA, stroke mimics, transient ischemic attacks (TIAs), and minor strokes (defined as baseline NIHSS score ≤ 4 points) were further excluded. At hospital arrival, eligible patients received reperfusion therapies, stroke unit admission, and etiologic workup according to their treating physician's discretion. Stroke etiology was assessed with the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification.[15] Outcome was evaluated 3 months after stroke with the modified Rankin Scale (mRS). A score on the mRS >2 points was considered poor outcome.

A second cohort, including 62 patients with a diagnosis of stroke mimic included in the Stroke-Chip study,[13] was selected based on sample availability.

Standard Protocol Approvals, Registrations, and Patient Consents

The study protocol was approved by the Vall d'Hebron Clinical Research Ethics Committee (PR [AG]157/2011) and all patients or their relatives gave written informed consent.

Blood Collection and Biomarker Measurement

Blood samples were collected at hospital admission before IV tPA was started. Blood was collected into EDTA tubes and centrifuged at 1,500 *g* for 15 minutes at 4°C and plasma aliquots were frozen at −80°C until biomarker measurement. Assays for GFAP from BioVendor–Laboratorni medicina a.s.

(Brno, Czech Republic), RBP-4 and endostatin from R&D Systems Inc. (Minneapolis, MN), and NT-proBNP from Roche Diagnostics GmbH (Mannheim, Germany) were performed according to the manufacturer's instructions by individuals who were blinded to the clinical diagnosis. All samples were tested in duplicate and the mean coefficient of variation (CV) was <20%. In a subgroup consisting of 68 patients (34 IS and 34 ICH) matched one-to-one by age, sex, and NIHSS score, GFAP was determined with a Simoa high-sensitivity assay from Quanterix. In the stroke mimic cohort, GFAP was measured with just the Simoa high-sensitivity assay.

Point-of-Care Setup

For fast measurement of the selected markers, lateral-flow POC devices were set up for RBP-4 (Mologic Ltd., Thurleigh, UK) and NT-proBNP (Shenzhen Kang Sheng Bao Bio-Technology Co., Ltd. [KSB], Hong Kong). No commercially available lateral-flow immunoassay with the requested sensitivity was found for GFAP. According to the manufacturer's instructions, 80 μL of serum and 80 μL of plasma were added into the RBP-4 and NT-proBNP test device sample wells, respectively. Optical densities (ODs) of these biomarkers were read using the Cube-Reader from Optricon (optricon.de/cube-reader.php). Both lateral-flow devices and optical readers are commercially available. Afterwards, to extrapolate the OD readings to concentration values and to determine optimal dilutions, standard curves were generated; for RBP-4, serial dilutions of an already known high-concentration RBP-4 serum sample were generated. For NT-proBNP, recombinant NT-proBNP protein and plasma free NT-proBNP (Hytest, Turku, Finland) were used to obtain the different points of the curve. For RBP-4 lateral-flow POC devices, the selected dilution for serum samples was 1/200. For NT-proBNP, the samples were not diluted.

To evaluate the performance of the test, concentrations were first compared between the available ELISA measurements of patients with IS from the previous cohort and lateral-flow immunoassays in 6 serum RBP-4 and 11 plasma NT-proBNP samples. Whole blood and plasma levels were also compared using 16 (RBP-4) and 15 (NT-proBNP) patients with new acute IS.

Statistical Analysis

Statistical analyses were conducted with Statistical Packages for Social Sciences (SPSS), version 22, and RStudio, version 1.1.447. Graphs were displayed with Prism version 8.1.2 (227) and the ggplot2 library from the same RStudio version. Data are expressed as numbers (percentages) for categorical variables. All continuous variables were non-normally distributed (Kolmogorov-Smirnoff test, $p < 0.05$) and, therefore, are expressed as the median (interquartile range). Comparisons were performed between IS and ICH. Univariate analysis was performed using the χ^2 test for categorical variables and the Mann-Whitney *U* test for continuous variables. A p value < 0.05 was considered statistically significant. To assess the role of biomarkers as independent predictors of ICH, logistic regression analysis was performed, considering at the first step biomarkers and clinical variables differentiating IS and ICH at a p value < 0.1. To develop 2-biomarker panels, the most accurate cutoff points were selected for the best sensitivity at 100% specificity for IS (thereby not allowing any patient with ICH to be misidentified as having IS). For the development of 3-biomarker panels, the most informative biomarker was dichotomized first into the cutoff with the highest accuracy to exclude most patients with ICH. In a second step, the selection of the cutoffs for the remaining markers was performed as for the case of 2-biomarker panels (best sensitivity at 100% specificity for IS). In addition, we used support vector machines to maximize the rate of IS classification while maintaining 100% specificity.[16] A radial kernel was used, and the parameters were selected from a grid with several pairs of c and sigmas. The criterion to select the best of them was to maximize the accuracy of classification. The pair of parameters selected was c = 100 and sigma = 0.05. To obtain a classifier with 100% specificity for ICH, the decision value should have to be increased at each point to 0.71. Finally, patients with IS correctly identified with the biomarker panel were compared with those not correctly identified in terms of clinical variables and outcome. To evaluate the performance of the different lateral-flow POC test (POCT) devices, correlations were assessed with the Spearman rank test. In addition, Bland-Altman plots were used to assess the agreement between immunoassays and POCTs.

Data Availability

Pseudonymized data can be made available to qualified researchers on request.

Results

Patients

During the study period, out of 592 patients with acute stroke visiting the emergency department, 365 were attended within the first 4.5 hours after symptom onset. After the exclusion of 56 stroke mimics, 26 TIAs, and 85 minor strokes, 161 patients with IS and 37 patients with ICH were available for study inclusion. In 8 patients, plasma samples were not available. Therefore, 190 patients were included; stroke subtype diagnosis was IS in 155 patients and ICH in 35 patients. An additional patient with IS was excluded for presenting with a spontaneous hemorrhagic transformation on baseline CT; therefore, the final number of patients included was 189. Of the included patients with IS, 87 out of 154 (56.9%) were treated with IV tPA and 43 (28.5%) were treated with MT. As shown in table 1, patients with ICH had higher admission blood pressure and tended to have higher stroke severity at admission. Patients with IS had higher rates of atrial fibrillation (AF) and tended to be more frequently female. As expected, patients with ICH had worse functional outcomes and higher mortality at 3 months.

Biomarkers

As shown in figure 1, baseline levels of NT-proBNP, endostatin, RBP-4, and GFAP were different between the stroke subtypes, with higher levels of GFAP in ICH (0.045 [0.045–0.059] ng/mL

Table 1 Baseline Characteristics of the Study Cohort and Differences Between Ischemic Stroke (IS) and Intracerebral Hemorrhage (ICH)

	All (n = 189)	ICH (n = 35)	IS (n = 154)	*p* Value
Age, y	81 (71–85)	82 (72–85.5)	81 (70–85)	0.462
Sex (female)	105 (55.6)	15 (42.9)	90 (58.4)	0.094[a]
Smokers	17 (9.0)	1 (2.9)	16 (11.0)	0.203
Alcohol	9 (4.8)	1 (2.9)	8 (5.5)	0.999
Hypertension	143 (75.7)	28 (80)	115 (75.4)	0.545
Dyslipidemia	86 (45.5)	17 (48.6)	69 (44.8)	0.686
Diabetes mellitus	53 (28)	6 (17.1)	47 (30.5)	0.112
Previous stroke	37 (19.6)	4 (11.4)	33 (21.4)	0.178
AF	58 (30.7)	5 (14.3)	53 (34.4)	0.020[b]
CAD	27 (14.3)	3 (8.6)	24 (15.6)	0.285
PAD	8 (4.2)	0 (0)	8 (5.2)	0.355
SBP, mm Hg	152.5 (137–168)	160 (152–197)	149 (133–163)	0.001[b]
DBP, mm Hg	78 (68–90)	84 (71–100)	77 (67–90)	0.020[b]
Glucose, mg/dL	129 (108–165.5)	136 (114–177.5)	127 (106–162.5)	0.225
Admission NIHSS	14 (9–19)	17 (11–20)	13 (9–18)	0.081[a]
3-month poor outcome[c]	120 (63.5)	31 (88.6)	89 (57.8)	0.001[b]
3-month all-cause mortality	56 (29.6)	18 (51.4)	38 (24.7)	0.003[b]

Abbreviations: AF = atrial fibrillation; CAD = coronary artery disease; DBP = diastolic blood pressure; NIHSS = NIH Stroke Scale; PAD = peripheral artery disease; SBP = systolic blood pressure.
The results are expressed as n (%) for categorical variables and median (interquartile range) for continuous variables.
[a] $p < 0.1$.
[b] $p < 0.05$.
[c] Defined as modified Rankin Scale score ≥2.

vs 0.045 [0.045–0.045] ng/mL, $p < 0.0001$) and higher levels of RBP-4 (34.4 [26.0–40.0] μg/mL vs 29.2 [25.1–35.7] μg/mL, $p = 0.053$), NT-proBNP (789 [194.5–2,357.0] pg/mL vs 365.8 [157.9–713.2] pg/mL, $p = 0.022$), and endostatin (213.4 [158.0–264.3] vs 181.9 [150.1–217.8] ng/mL, $p = 0.046$) in IS. Of note, only 15 (22%) patients had GFAP values over the lower detection limit of the conventional ELISA (1 IS and 14 ICH).

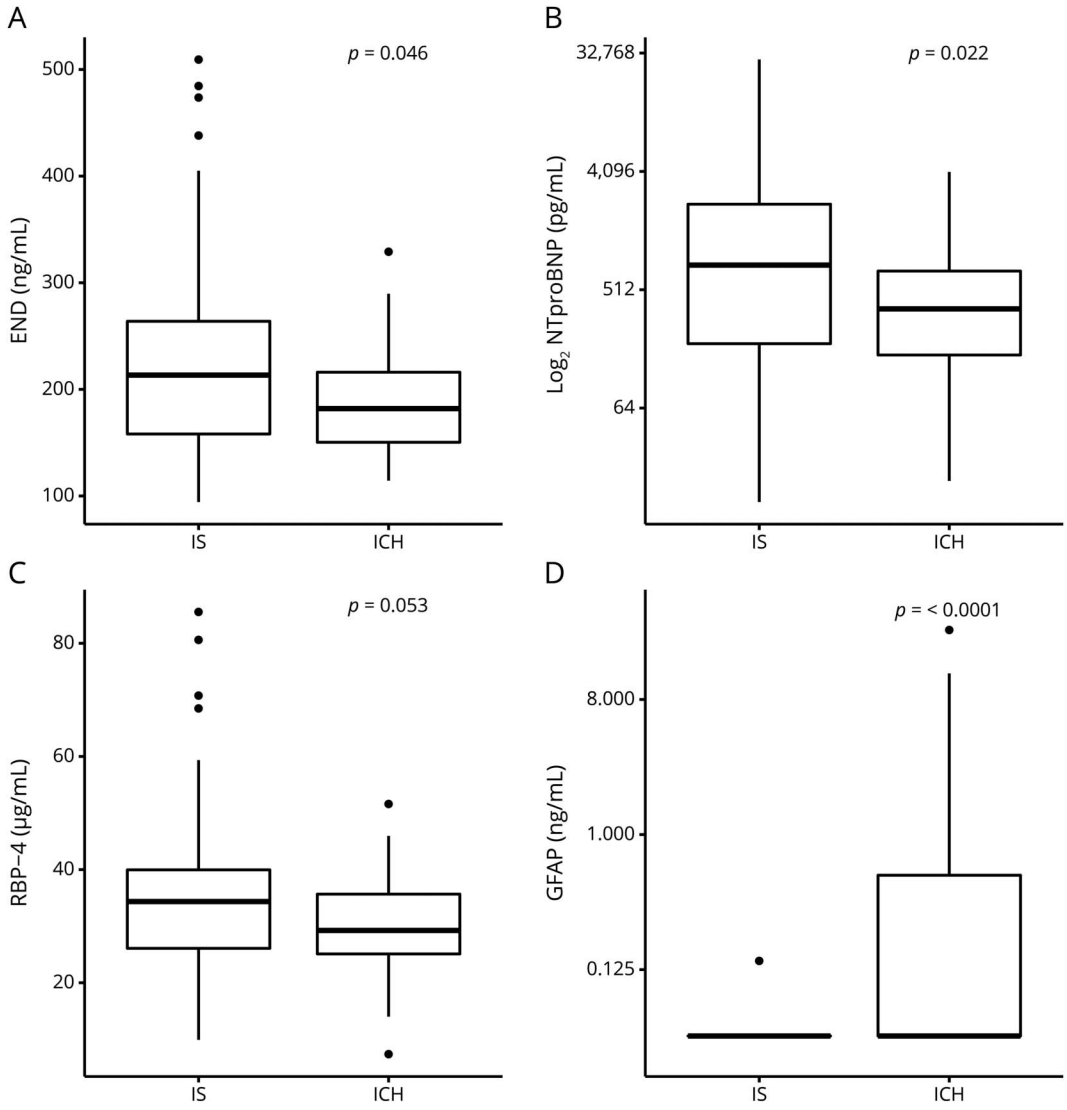
Logistic Regression Models

Cutoffs with the highest accuracies were used to assess the independent predictive value of each biomarker for the differentiation of IS vs ICH: GFAP = 0.05 ng/mL (37.1% sensitivity, 99.4% specificity); RBP-4 = 31.8 μg/mL (56.5% sensitivity, 68.6% specificity); NT-proBNP = 1,468.5 pg/mL (33.8% sensitivity, 94.1% specificity); endostatin = 222.57 ng/mL (46.1% sensitivity, 79.4% specificity). After including blood biomarkers in the model, baseline NIHSS score (odds ratio [OR] 1.16 [1.04–1.28], $p = 0.007$) was the only clinically independent predictor of ICH subtype, together with GFAP >0.05 ng/mL (OR 154.22 [7.04–3,380.39], $p = 0.001$) and NT-proBNP >1,468.5 pg/mL (OR 0.02 [0.01–0.58], $p = 0.022$), while RBP-4 >31.8 μg/mL disclosed a trend (OR 0.29 [0.08–1.08], $p = 0.064$). Endostatin was not an independent predictor (OR 0.75 [0.15–3.80], $p = 0.730$); therefore, it was not further used to develop biomarker panels.

Selection of Biomarker Panels for IS Detection

The diagnostic yield of the combination of NT-proBNP, RBP-4, and GFAP to differentiate stroke subtypes was evaluated with combined cutoffs selected for the best sensitivity at 100% specificity for IS. This 100% specificity would allow a safe treatment of IS in selected patients, avoiding the possibility of giving a harmful therapy to a patient with the wrong subtype (e.g., treating a patient with ICH with tPA). GFAP was not useful given that most of the patients with IS had no detectable values of GFAP. The best combination for IS diagnosis was RBP-4 >52 μg/mL and NT-proBNP >4,062 pg/mL, providing a 20% sensitivity and 100% specificity for IS diagnosis (31 out of 154 patients with IS detected, figure 2A). Positive and negative predictive values for the panel were 100% and 21.7%, respectively. A post hoc analysis using support vector machine analysis was able to improve the sensitivity to 29.7% at the cost of a modest accuracy of 42% (figure 2B).

Figure 1 Biomarker Levels Among Patients With Ischemic Stroke (IS) and Patients With Intracerebral Hemorrhage (ICH)



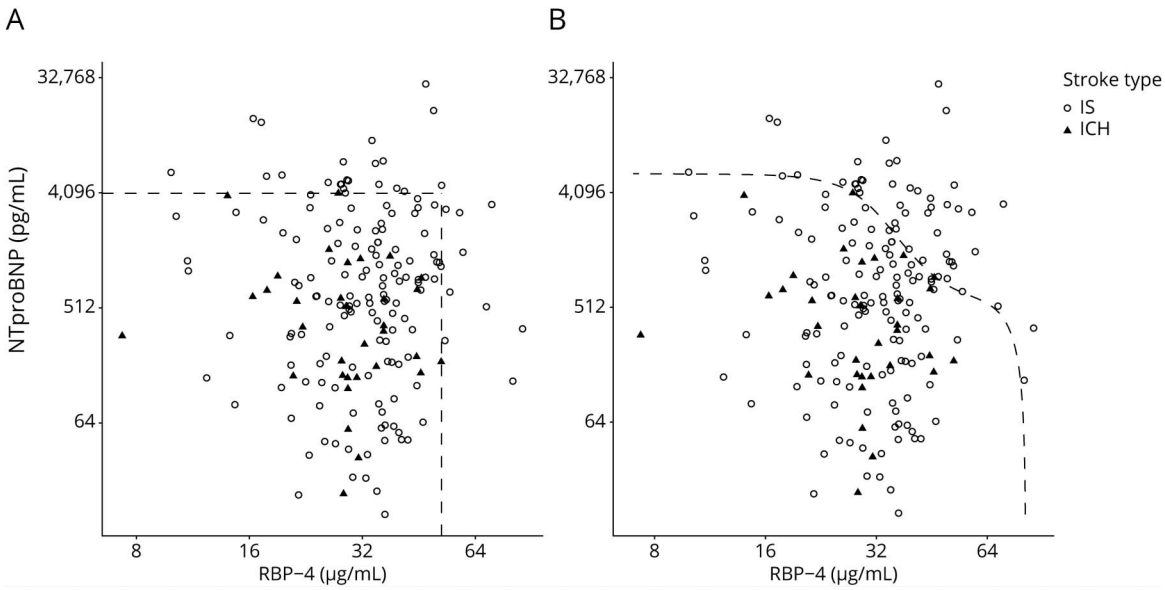
Boxplots represent the median and interquartile range of endostatin (A), N-terminal proB-type natriuretic peptide (NT-proBNP) (B), retinol binding protein 4 (RBP-4) (C), and glial fibrillary acid protein (GFAP) (D) levels among patients with IS (n = 154) and ICH (n = 35).

GFAP High-Sensitivity Quanterix Assay Subanalysis

An additional determination using the Quanterix assay was performed in a subgroup of 68 patients from the same cohort, including 34 IS and 34 ICH, matched by age, sex, and NIHSS score. One patient with ICH was excluded due to a CV of 23%. No significant clinical differences were noted between IS and ICH except for higher rates of blood pressure in patients with ICH and a higher prevalence of AF in patients with IS (data not shown). In contrast to conventional ELISA, GFAP values were obtained in all patients with the Quanterix assay. A moderate but significant correlation was noted between Quanterix and ELISA assays ($R = 0.635$, $p < 0.0001$). Patients with ICH had higher GFAP levels than those with IS (1,699.6 [411.1–10145.4] pg/mL vs 186.3 [132.8–280.2], $p < 0.0001$), and this difference was present when only patients with previously undetectable levels were included in this comparison (n = 31 IS and 21 ICH) (751.5 [237.9–1,652.9] pg/mL vs 185.3 [125.5–276.8], $p < 0.0001$).

The best combination for 100% specificity for IS was performed following a 2-step approach: first, patients with high levels of GFAP (>325 pg/mL) were removed (6 IS and 27 ICH). In the remaining patients, the combination of NT-proBNP >1,305 pg/mL and RBP-4 >38 µg/mL disclosed a 51.5% sensitivity (17 out of 33 patients with IS) for IS diagnosis (figure 3). Positive and negative predictive values were 100% and 68% for the panel, respectively.

Figure 2 Biomarker Panel for Ischemic Stroke (IS) Diagnosis in the Whole Cohort

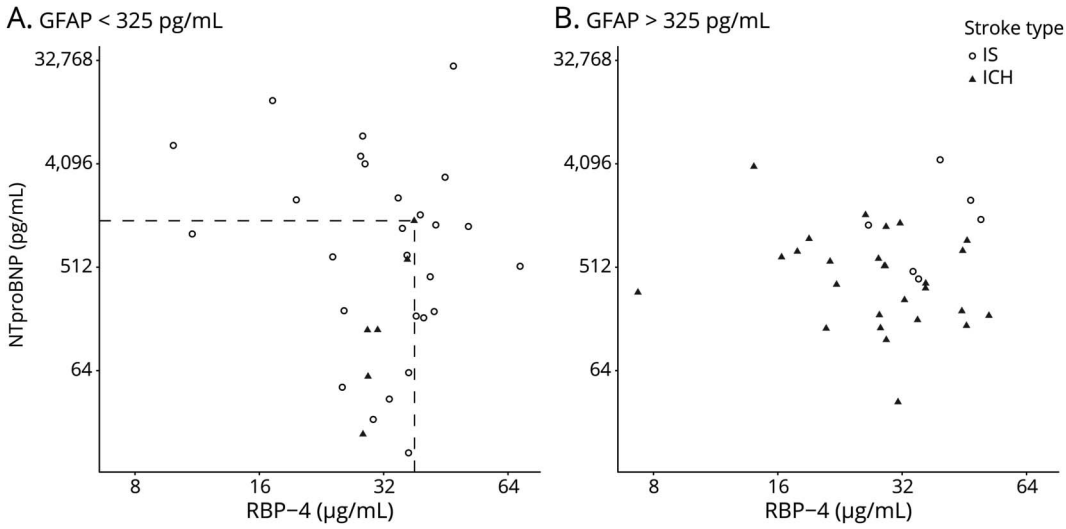


The figure represents each patient's levels of retinol binding protein 4 (RBP-4) and N-terminal proB-type natriuretic peptide (NT-proBNP) according to stroke diagnosis. Patients with IS (n = 154) are represented with clear circles and patients with intracerebral hemorrhage (ICH) (n = 35) with dark triangles. (A) Cutoffs of RBP-4 (52 µg/mL) and NT-proBNP (4,062 pg/mL) are represented with dashed lines. NT-proBNP values are given as log10 for a better interpretation. (B) Support vector machine analysis in the whole cohort to differentiate between IS and ICH. The figure represents NT-proBNP and RBP-4 levels in the whole cohort of IS and ICH, represented as light circles and dark triangles, respectively. The dashed line represents the criterion where a patient can be classified as having IS or ICH. The decision value was changed to −0.71 to achieve 100% specificity. With this method, we were able to identify 46/155 (29.7%) patients with IS (above the dashed line).

Comparisons Between Selected and Nonselected Patients

For the first biomarker panel (RBP-4 >52 µg/mL, NT-proBNP >4,062 pg/mL), the identified patients with IS were compared with those not identified with the biomarker combination. Identified patients had a higher percentage of diabetes mellitus and AF and higher rates of cardioembolic etiology. Despite no differences in baseline stroke severity,

Figure 3 Biomarker Panel for Ischemic Stroke (IS) Diagnosis in the Substudy With the High-Sensitivity Assay for Glial Fibrillary Acid Protein (GFAP)



The figure represents each patient's levels of retinol binding protein 4 (RBP-4) and N-terminal proB-type natriuretic peptide (NT-proBNP) according to stroke diagnosis and GFAP levels. Patients with IS (n = 34) are represented with light circles and patients with intracerebral hemorrhage (ICH) (n = 33) are represented with dark triangles. Panels represent patients with GFAP levels under (A) and over (B) the cutoff of GFAP of 325 pg/mL, respectively. Dashed lines in A represent cutoffs of NT-proBNP (1,305 pg/mL) and RBP-4 (38 µg/mL). NT-proBNP values are given as log10 for better visualization.

outcome and mortality were worse in the group of patients correctly identified. Those patients also tended to be older, to have higher glucose levels, and to be less prone to receive tPA (table 2). Regarding the substudy with the GFAP high-sensitivity assay, the identified patients were older and more prominently female, with a lower frequency of prior strokes. There were no differences in the rate of AF or diabetes; however, identified patients had higher baseline glycemia. No differences were found regarding mortality, but again, poor outcome at 3 months was more frequent among patients identified with the biomarker panel (data not shown).

Comparison With Stroke Mimics

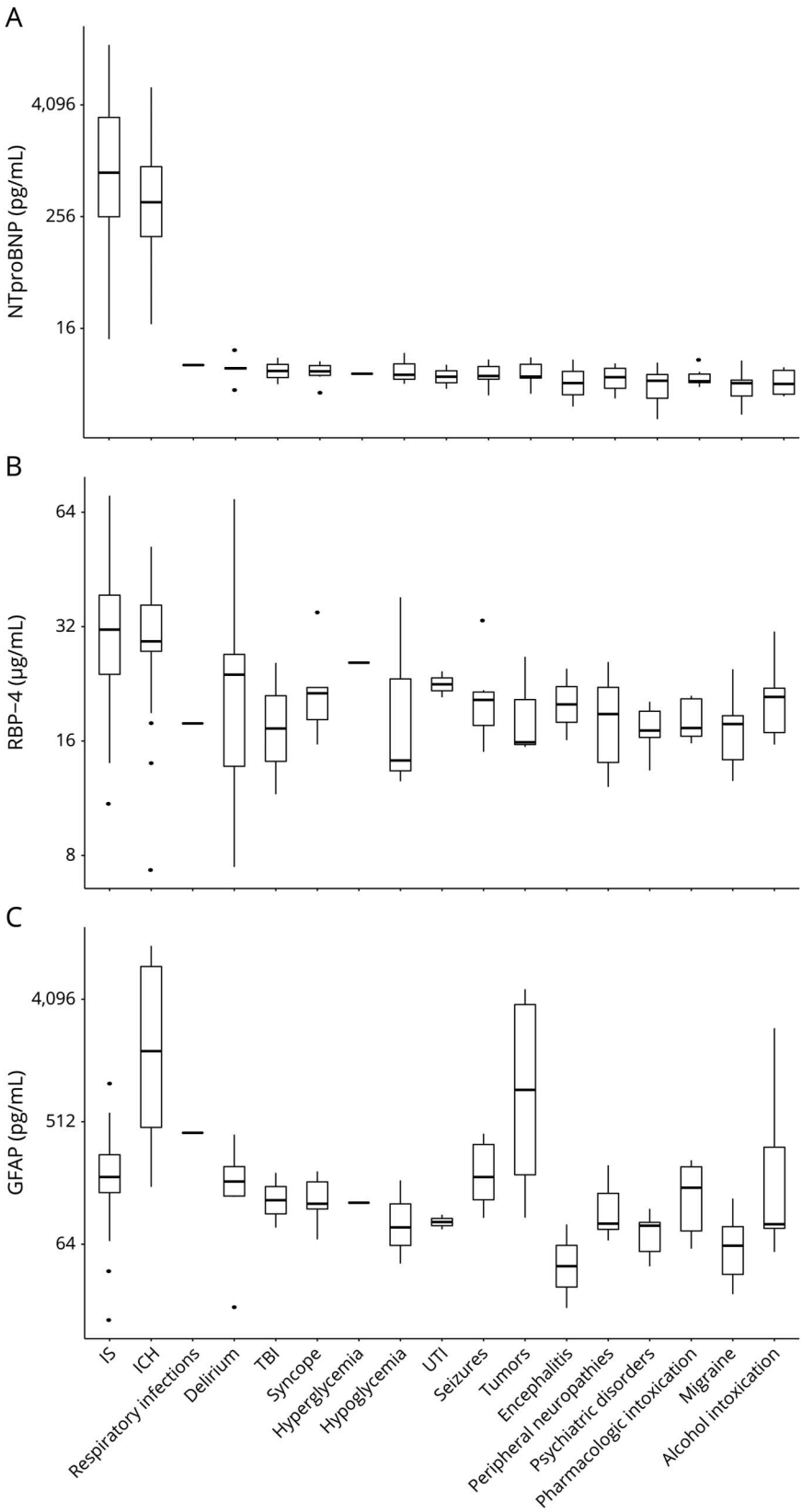
The cohort including 62 stroke mimics was also considered regarding the biomarker panel. With the first panel (RBP-4 >52 μg/mL + NT-proBNP >4,062 pg/mL), just 1 out of 62 patients (1.6%) was identified as having IS. With the second panel (GFAP <325 pg/mL + NT-proBNP >1,305 pg/mL + RBP-4 >38 μg/mL), 2 out of 62 stroke mimics (3.2%) were identified as having acute strokes. Regarding specific subtypes of stroke mimics, all types of mimics had lower NT-proBNP and RBP-4 than patients with IS. Regarding GFAP, the only type of mimic with elevated GFAP was brain tumors, as expected (figure 4).

Table 2 Comparison Between Patients With Ischemic Stroke Identified and Not Identified by the Combination of Retinol Binding Protein-4 >52 μg/mL and N-Terminal ProB-Type Natriuretic Peptide >4,062 pg/mL in the Whole Cohort

	Identified (n = 31)	Not identified (n = 123)	*p* Value
Age, y	83 (78–85)	79 (68–84)	0.054[a]
Sex (female)	22 (71.0)	68 (55.3)	0.113
Smokers	3 (9.7)	13 (11.3)	0.999
Alcohol	3 (9.7)	5 (4.4)	0.368
Hypertension	26 (83.9)	89 (73.0)	0.209
Dyslipidemia	11 (35.5)	58 (47.2)	0.243
Diabetes mellitus	14 (45.2)	33 (26.8)	0.048[b]
Previous stroke	8 (25.8)	25 (20.3)	0.506
AF	18 (58.1)	35 (28.5)	0.002[b]
CAD	7 (22.7)	17 (13.8)	0.268
PAD	2 (6.5)	6 (4.9)	0.664
SBP, mm Hg	148 (129–166)	149 (136–162)	0.513
DBP, mm Hg	70 (61.5–81)	78.5 (68.5–90)	0.068[a]
Glucose, mg/dL	136 (122.5–166.5)	124 (103–160)	0.085[a]
Admission NIHSS	15 (11–19)	13 (9–18)	0.284
ASPECTS	10 (8.5–10)	10 (9–10)	0.725
Time to tPA, h	2.2 (1.4–3.0)	1.9 (1.4–2.9)	0.729
Rate of thrombolysis	13 (41.9)	74 (60.7)	0.060[a]
CE stroke	22 (71.0)	60 (48.8)	0.027[b]
HT	7 (28.0)	22 (28.2)	0.984
sICH	2 (6.5)	6 (5.0)	0.666
3-month poor outcome[c]	24 (77.4)	65 (52.8)	0.013[b]
3-month all-cause mortality	15 (48.4)	23 (18.7)	0.001[b]

Abbreviations: AF = atrial fibrillation; ASPECTS = Alberta Stroke Program Early CT Score; CAD = coronary artery disease; CE = cardioembolic; DBP = diastolic blood pressure; HT = hemorrhagic transformation (any visible hemorrhagic transformation on CT scan); NIHSS = NIH Stroke Scale; PAD = peripheral artery disease; SBP = systolic blood pressure; sICH = symptomatic intracerebral hemorrhage (any HT visible in CT scan with neurologic deterioration: increase in 4 points or more in the NIHSS); tPA = tissue plasminogen activator.
Results are expressed as n (%) for categorical variables and median (interquartile range) for continuous variables.
[a] $p < 0.1$.
[b] $p < 0.05$.
[c] Defined as modified Rankin Scale score ≥2.

Figure 4 Biomarker Values in Stroke-Mimic Patients



Boxplots represent the median and interquartile range of N-terminal proB-type natriuretic peptide (NT-proBNP) (A), retinol binding protein 4 (RBP-4) (B), and glial fibrillary acid protein (GFAP) (C) levels among patients with IS (n = 154), patients with intracerebral hemorrhage (ICH) (n = 35), and those with stroke mimics (n = 64) divided by each different condition. TBI = traumatic brain injury; UTI = urinary tract infection.

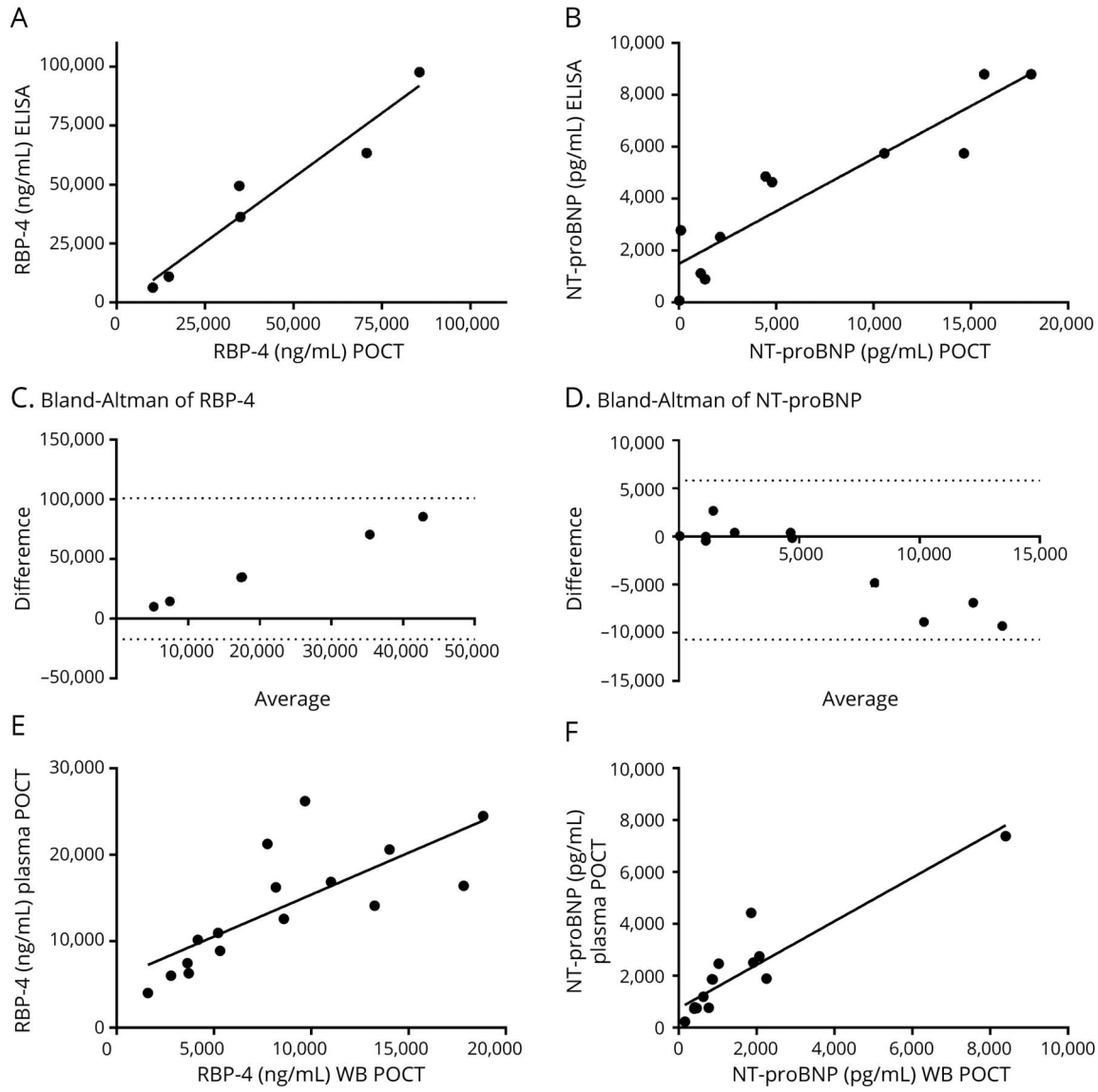
Point-of-Care Setup

A schematic representation of the POCT devices can be accessed online through the Biofast website at biofast.technology/en/devices/. For both biomarkers, measurements were simple and feasible within the specified times. The Bland-Altman plot represented in figure 5 shows the differences between concentrations measured using the ELISA method and the POCT devices. All measures were within the limits of agreement, showing that both methods were positively correlated. In addition, for both biomarkers, the correlations obtained between ELISA and lateral-flow POCT devices were significantly positive (RBP-4: R = 0.943, $p < 0.005$; NT-proBNP: R = 0.916, $p < 0.0001$, figure 5). Similarly, reproducibility between whole-blood and plasma samples was evaluated. For this purpose, whole-blood and plasma samples obtained from 16 (RBP-4) and 15 (NT-proBNP) patients with new acute IS were compared using Spearman correlation. The results represented in figure 5 show a positive correlation between the 2 different matrixes (RBP-4: R = 0.757, $p < 0.0001$; NT-proBNP: R = 0.916, $p < 0.0001$).

Discussion

The present study validates panels of blood-based biomarkers that were able to identify selected patients with acute IS among patients presenting with suspected stroke within the

Figure 5 Correlation Plots Between ELISA and Point-of-Care (POC) Assays



The figure shows the correlation plots for retinol binding protein 4 (RBP-4) (n = 6; A) and N-terminal proB-type natriuretic peptide (NT-proBNP) (n = 11; B) between ELISA and lateral-flow POC test (POCT) assays and Bland-Altman plots for the 2 measures of RBP-4 (C) and NT-proBNP (D). The x-axis represents the average of the 2 measures (ELISA and POCT) and the y-axis represents the difference. Horizontal discontinuous lines represent the average difference and the 95% limits of agreement. Correlation between POCT measures in plasma and whole blood for RBP-4 (n = 16) and NT-proBNP (n = 15) is represented in E and F, respectively.

4.5 hours time window for IV tPA, suggesting that prehospital thrombolysis might be safe in selected cases without the need for prehospital imaging.

Previous studies on the same indication have failed to identify a biomarker panel able to accurately differentiate between IS and ICH. Glial biomarkers such as GFAP have been proposed as interesting candidates given their different release patterns in IS and ICH, with earlier peaks within the first hours after ICH and a more delayed release pattern in IS.[17] However, even when those biomarkers have proven (especially in the case of GFAP) usefulness in the identification of patients with ICH due to a high specificity for this stroke subtype,[10–12] the absence of specific acute therapies for ICH has precluded its clinical use so far. Other studies focusing on biomarker panels have found maximum accuracy levels of approximately 80% for this differentiation, even after the inclusion of clinical variables such as the NIHSS score in the predictive models.[13] In the present study, we focused on finding a biomarker combination that was able to provide a very specific IS diagnosis, thereby avoiding false-positives (any ICH diagnosed as an IS), at the expense of some false-negatives. If confirmed in future studies, this strategy might allow prehospital thrombolysis in selected IS cases. Despite the modest sensitivity raised by the panels, the chance for patients with false-negative diagnoses to receive tPA would not be compromised, as those patients would be transferred to a hospital to receive standard imaging-based diagnosis, resulting in no delay in hospital arrival due to the speed of the POCTs.

In the present study, rather than identifying new biomarkers, we focused on a selected combination of biomarkers previously described as potentially useful to differentiate IS from ICH. NT-proBNP, endostatin, and RBP-4 were previously described as elevated in IS compared with ICH,[12,13] perhaps reflecting a higher contribution of IS-related risk factors, such as AF and heart failure, for the case of NT-proBNP. Additionally, RBP-4 has been described as being associated with diabetes mellitus and insulin resistance.[18] When we compared patients with high levels of both NT-proBNP and RBP-4 (those patients correctly identified with the combination) with the remaining patients with stroke, we found that those patients had a higher burden of these risk factors (AF, diabetes). However, these patients also had poorer outcomes, specifically poor functional outcome for both panels and worse mortality for panel 1, despite no differences in stroke severity, Alberta Stroke Program Early CT Score, or time to treatment. The lack of differences regarding mortality for panel 2 is likely due to the small number of deaths in IS in this substudy (10 deaths at 3 months). Although we do not have a clear hypothesis for these findings, the possibility to offer these patients earlier therapies seems promising to improve their outcomes.

For the case of GFAP, as expected, the biomarker did not prove to be useful in the selection of a subgroup of IS with high specificity, given that GFAP was not detectable with conventional assays in most of the patients with IS. These findings are in line with those of previous literature.[19] However, with the use of high-sensitivity assays, we were able to differentiate between IS and ICH even in those patients in whom GFAP was not detectable with conventional assays, which manifests the importance of the detection of minimal amounts of GFAP. This fact allowed us to use less restrictive cutoffs for the other markers, thereby improving the sensitivity of the panel. The main problem with this marker is that such assays are difficult to implement in conventional lateral-flow immunoassays, which is necessary for the present indication.

If our findings could be confirmed in future cohorts, with prehospital blood samples obtained, the present study may be a revolution in the field of acute stroke treatment. Prehospital thrombolysis has shown feasibility only when ICH is ruled out with a portable CT scan,[8] and the benefits of treating patients sooner with mobile stroke units are important.[9] However, the costs associated with this expensive strategy are probably impossible to be extended globally, and the question of its cost-effectiveness has not been answered so far.[20]

A biomarker panel able to safely identify a subgroup of patients with IS would allow prehospital thrombolysis in selected cases, and this strategy could be adopted all around the world, even in low- and middle-income countries, where the lack of available neuroimaging represents the main obstacle for acute-phase treatment. However, the field that the present study introduces is not free of controversies. In addition to being able to differentiate between IS and ICH, neuroimaging provides more valuable information, such as risk factors for hemorrhagic transformation (periventricular white matter disease, microbleeds in the case of MRI) or subacute infarctions, as the time window is not always reliable. Furthermore, in most health care systems, prehospital care is performed by paramedics, who make these decisions in the face of a long list of contraindications for tPA administration. In Spain, prehospital stroke care is usually carried out by trained emergency physicians, who usually perform prehospital scales to identify patients with a high chance of large vessel occlusion, such as the Rapid Arterial Occlusion Evaluation (RACE) scale.[21] Even in the absence of on-board physicians, acute stroke care has proven to be feasible when administered by trained paramedics, as was demonstrated by the FAST-MAG (Field Administration of Stroke Therapy–Magnesium) clinical trial.[22] As an alternative, telemedicine systems are usually incorporated in acute stroke care and might support an alternative pathway similar to the one proposed. Even when our results provide 100% specificity for IS, in a future use, we cannot rule out the possibility of the misdiagnosis of a case of ICH and, therefore, the administration of tPA to a patient with ICH. Even in this case, our strategy could have an overall risk–benefit profile, as has been suggested.[23] However, legal implications preclude these statements and should be carefully considered. A future scenario in which tPA could be administered to patients with IS in the absence of

neuroimaging, based on just blood biomarker information, will require more replication studies with validated CE-marked POCTs, ideally in a prehospital scenario.

In this sense, experimental literature points to an effect of tPA less catastrophic than what could be anticipated, suggested by previous experiments in rodents.[24,25] In any case, these results need to be carefully interpreted, as an animal model of ICH does not carry other risk factors for catastrophic bleeding, such as white matter hyperintensities, uncontrolled hypertension, or hyperglycemia.

Our study presents several limitations. First, the sample size is relatively small, especially for the substudy with the high-sensitivity GFAP assay, and lacks a replication cohort. However, all patients with high GFAP levels determined with conventional ELISA and most patients with ICH from the original study were included. Regarding the replication cohort, the ongoing BIOFAST study (Biomarkers for Initiating Onsite and Faster Ambulance Stroke Therapies) (biofast.technology/) is enrolling patients with suspected acute stroke within 4.5 hours with no contraindications to tPA, collecting prehospital samples, and measuring NT-proBNP and RBP-4 with POCTs. This cohort, which will also include stroke mimics, will be an excellent opportunity to validate these findings. Second, patients on anticoagulants were included in the study, even when anticoagulant therapy constitutes a contraindication for the administration of tPA, unless the international normalized ratio might be obtained prior to tPA administration in patients on vitamin K antagonists. In this sense, we performed a sensitivity analysis (data not shown) excluding 43 patients on anticoagulants, and the results in terms of sensitivity and specificity were barely changed. Third, the fact that the cutoffs used were determined in this study, rather than the use of previously described cutoffs, results in bias and, therefore, might reduce the external generalizability of the present results. However, previous studies differentiating IS and ICH included cutoffs with the highest accuracy for the comparison instead of cutoffs for 100% specificity. In addition, these cutoffs could change with the use of different assays, as well as including new markers in the panels. In this sense, future studies are needed with validated assays to specify which cutoffs provide the maximum sensitivity for IS with 100% specificity. Ideally, those studies should be conducted at mobile stroke units attending stroke codes, and including head-to-head comparisons of imaging and biomarker results in the field. This might be challenging, because these studies would include stroke mimics and patients with stroke with blood samples drawn at very short times from stroke onset, a real-life scenario where the accuracy of the proposed biomarkers has not been tested.

Our study provides a biomarker panel that might be useful to safely identify selected patients with IS who might receive tPA even in the absence of neuroimaging. This biomarker panel might open a new field in acute IS care if confirmed in future, ongoing studies with validated POC devices and prehospital samples.

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Disclosure

Dr. Bustamante, A. Penalba, Dr. Orset, and Dr. Azurmendi report no disclosures. Dr. Llombart is a coinventor of a patent covering the use of blood biomarkers to differentiate ischemic from hemorrhagic stroke to guide reperfusion therapies. Dr. Simats, E. Pecharroman, O. Ventura, Dr. Ribó, Dr. Vivien, and Dr. Sanchez report no disclosures. Dr. Montaner is a co-inventor of a patent covering the use of blood biomarkers to differentiate ischemic from hemorrhagic stroke to guide reperfusion therapies. Go to Neurology.org/N for full disclosures.

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Appendix *(continued)*

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Jean Charles Sanchez, PhD	University of Geneva, Switzerland	Revised the manuscript for intellectual content
Joan Montaner, MD, PhD	Vall d'Hebron Institute of Research (VHIR), Barcelona, Spain	Design and conceptualized study, revised the manuscript for intellectual content

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