Blood Biomarkers for the Early Diagnosis of Stroke
The Stroke-Chip Study

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Background and Purpose—Stroke diagnosis could be challenging in the acute phase. We aimed to develop a blood-based diagnostic tool to differentiate between real strokes and stroke mimics and between ischemic and hemorrhagic strokes in the hyperacute phase.

Methods—The Stroke-Chip was a prospective, observational, multicenter study, conducted at 6 Stroke Centers in Catalonia. Consecutive patients with suspected stroke were enrolled within the first 6 hours after symptom onset, and blood samples were drawn immediately after admission. A 21-biomarker panel selected among previous results and from the literature was measured by immunoassays. Outcomes were differentiation between real strokes and stroke mimics and between ischemic and hemorrhagic strokes. Predictive models were developed by combining biomarkers and clinical variables in logistic regression models. Accuracy was evaluated with receiver operating characteristic curves.

Results—From August 2012 to December 2013, 1308 patients were included (71.9% ischemic, 14.8% stroke mimics, and 13.3% hemorrhagic). For stroke versus stroke mimics comparison, no biomarker resulted included in the logistic regression model, but it was only integrated by clinical variables, with a predictive accuracy of 80.8%. For ischemic versus hemorrhagic strokes comparison, NT-proBNP (N-Terminal Pro-B-Type Natriuretic Peptide) >4.9 (odds ratio, 2.40; 95% confidence interval, 1.55–3.71; $P<0.0001$) and endostatin >4.7 (odds ratio, 2.02; 95% confidence interval, 1.19–3.45; $P=0.010$), together with age, sex, blood pressure, stroke severity, atrial fibrillation, and hypertension, were included in the model. Predictive accuracy was 80.6%.

Conclusions—The studied biomarkers were not sufficient for an accurate differential diagnosis of stroke in the hyperacute setting. Additional discovery of new biomarkers and improvement on laboratory techniques seem necessary for achieving a molecular diagnosis of stroke. **(*Stroke*. 2017;48:2419-2425. DOI: 10.1161/STROKEAHA.117.017076.)**

Key Words: biomarkers ■ cerebral hemorrhage ■ diagnosis ■ hypertension ■ stroke

Stroke currently represents the fifth cause of death, causing 1 of every 20 deaths in the United States. On average, someone dies of stroke every 4 minutes.[1] Although for the case of intracerebral hemorrhage no treatment has demonstrated efficacy, intravenous tPA (tissue-type plasminogen activator) has shown usefulness in the treatment of acute ischemic stroke, being the benefit of this treatment highly dependent on time from symptom onset to administration.[2] Prehospital administration of tPA has shown feasibility,[3] although the existence of an intracerebral bleeding needs to be previously ruled out.

Received February 16, 2017; final revision received June 23, 2017; accepted June 27, 2017.

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Presented in part at the European Stroke Organization Conference, Barcelona, Spain, May 10–12, 2016.

The online-only Data Supplement is available with this article at http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.117.017076/-/DC1.

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For this issue, portable computed tomography scans have been used; however, a wide generalization of these expensive tools seems not feasible in the near future.

Beyond subtype differentiation, some conditions can simulate a stroke during the acute phase, namely stroke mimics. It has been reported that its frequency could be as high as 20% to 30% of the stroke code activations at the prehospital level.[4] Although some studies have found important rates of stroke mimics among those patients treated with tPA, this therapy has been demonstrated to be relatively safe in stroke mimics.[5] An accurate discrimination between these conditions might be helpful, not just in terms of safety but also in saving scarce resources, such as expensive emergency transfers or admission to stroke units.

Stroke diagnosis could be challenging within the hyperacute phase and is highly dependent on neuroimaging techniques, making prehospital diagnosis complicated. Contrary to acute coronary syndrome, stroke cannot be diagnosed by such a simple tool as an ECG. An alternative test achieving enough sensitivity and specificity in the discrimination between real strokes and stroke mimics, and also between both stroke subtypes, might be useful to guide prehospital stroke management, including patient's allocation and prehospital reperfusion therapies in the very early phase of stroke. Blood biomarkers represent an objective measurement of molecular characteristics and have been proposed as a tool to help in acute stroke diagnosis. A panel of biomarkers, including caspase-3, D-dimer, soluble receptor for advanced glycation end products, chimerin, secretagogin, and matrix metalloproteinase 9 (MMP-9), showed a predictive accuracy of 0.759 to differentiate between strokes and mimicking conditions within the first 24 hours in a study including 1005 patients with suspected stroke.[6]

With this background, we designed the Stroke-Chip study, with the aim of developing a panel of biomarkers to differentiate between real strokes and stroke mimics and between ischemic and hemorrhagic strokes in the acute phase.

Methods

Study Design and Clinical Protocol

The Stroke-Chip was an observational, prospective, multicentre study performed at the emergency departments of 6 Hospitals in Catalonia (Hospital Universitari Germans Trias i Pujol, Hospital Universitari Vall d'Hebron and Hospital Universitari de Bellvitge at Barcelona, Hospital Universitari Josep Trueta in Girona, Hospital Universitari Joan XXIII in Tarragona, and Hospital Verge de la Cinta in Tortosa), from August 2012 to November 2013. The study protocol was approved by each one of the Ethics Committees of the participating centers, and all patients or relatives signed the informed consent.

Inclusion criteria were age >18; suspected stroke at the time of first medical evaluation with persisting symptoms at the time of emergency room arrival; time from symptom onset to blood samples collection of <6 hours; blood collection previous to thrombolytic treatment; and signed informed consent. In cases of undetermined time of onset, the last time that the patient was known to be asymptomatic was considered as the time of stroke onset. The only exclusion criterion was impossibility to get blood samples. Patients were enrolled at Hospital arrival by neurologists. Patients in whom a certain diagnosis could not be obtained after 1 month of the index event were further excluded from the analyses.

After inclusion, clinical and radiological data were collected into standardized forms. Vitals (blood pressure, temperature) were collected at hospital admission. Stroke diagnosis was performed by trained neurologists at each center, according to the World Health Organization definition[7] and confirmed by neuroimaging. Stroke mimic diagnosis was supported with the ancillary tests deemed to be necessary in each case (ie, EEG, lumbar puncture). At the time of clinical diagnosis, researchers were not aware about biomarkers data. Symptoms severity was assessed with the National Institutes of Health Stroke Scale score by the attending neurologist.[8]

Biomarker Panel Selection

A panel, including 21 biomarkers, was selected for the present study. Some of the biomarkers were chosen from previous results of our groups: IL-17A (interleukin-17A), IL-2RG, IGFBP-3 (insulin-like growth factor-binding protein-3), tumor necrosis factor receptor-1 (TNF-R1), growth-related oncogene-α, FasL (Fas ligand) and βNGF (β-nerve growth factor; Methods I in the online-only Data Supplement), and Hsc70 (Heat shock 70 kDa protein-8; patent WO 2012152970 A1). Other biomarkers were chosen from literature (NT-proBNP [N-Terminal Pro-B-Type Natriuretic Peptide]), MMP-9, D-dimer and caspase-3,[6] IL-6,[9] cellular fibronectin (cFn),[10] Von Willebrand factor,[11] vascular adhesion protein-1 (VAP-1),[12] endostatin,[13] S100B,[14] apolipoprotein CIII,[15] neuron-specific enolase,[16] and neuron cell adhesion molecule (NCAM).[17]

Blood Samples Collection and Biomarker Measurement

Samples were drawn on admission. Blood was collected into EDTA tubes, centrifuged at 1500 g for 15 minutes at 4°C, and plasma aliquots were frozen at −80°C until biomarker measurement. Biomarker measurement was performed by different immunoassays listed at Methods II in the online-only Data Supplement. All assays were performed according to the manufacturer's instructions and blinded to clinical diagnosis. All samples were tested in duplicate, and the mean coefficient of variation was <20%. Interassay variation was determined by testing 2× in every plate a commercial internal control (Human serum type AB, male, from clotted, Sigma-Aldrich, cat number H6914). As most of the biomarkers showed coefficient of variation interassay >20%, biomarker values were log transformed with a base of 10. Moreover, when coefficient of variation remained >20% despite log transformation, biomarkers levels were standardized by dividing each value for the internal quality control in each plate.

Statistical Analysis

An interim analysis was planned with the first 500 patients enrolled, to select just the most informative biomarkers to be measured in the whole study cohort.

Statistical analyses were conducted with Statistical Packages for Social Sciences (SPSS), version 22. Data were expressed as number (percentage) for categorical variables and, depending on data distribution (assessed by the Kolgomorov–Smirnoff test), as mean±SD or median (interquartile range) for continuous variables. Comparisons were first performed between all strokes (ischemic and hemorrhagic) versus stroke mimics and secondly between ischemic and hemorrhagic strokes.

For each comparison, univariate analysis was performed, using the χ^2 test for categorical variables and, depending on data distribution, the Student t or the Mann–Whitney U test for continuous variables. A P value <0.05 was considered statistically significant. Biomarkers differentiating between the different conditions in univariate analysis were dichotomized at the cutoff point with the highest accuracy for each comparison, with receiver operating characteristic curves. Logistic regression models were developed for each comparison, including at the first step all biomarkers and clinical variables associated with the comparison at a P value <0.1 in univariate analysis. The

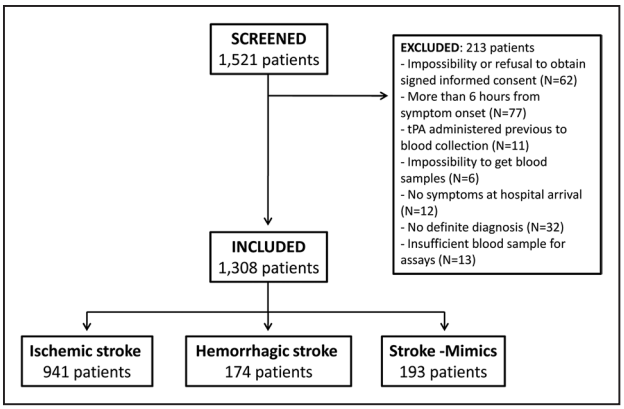

Figure. Study flow chart. tPA indicates tissue-type plasminogen activator.

accuracy of the models was evaluated with the area under the receiver operating characteristic curve.

Logistic regression models were derived from the interim analysis cohort and validated in the remaining patients. In addition, logistic regression models based on the whole cohort were also performed.

Results

Patients

From August 2012 to November 2013, 1521 patients were screened. Finally, 1308 patients were included. Reasons for exclusions are given in the Figure. Mean time from symptom onset to blood collection was 2:46±1:30 hours. Clinical diagnosis was ischemic stroke in 941 cases (71.9%), hemorrhagic stroke in 174 (13.3%), and stroke mimic in 193 (14.8%). A descriptive analysis of the whole cohort and the differences between groups is available in Table 1.

Biomarkers' Interim Analysis

The interim analysis was performed in the first 541 patients. Characteristics of this cohort are detailed in the Table I in the online-only Data Supplement. Seven out of the 21 biomarkers (βNGF, caspase-3, neuron-specific enolase, cFn, IL-2RG, IL-17A, and MMP-9) were not measured in the whole cohort because of being considered no discriminative at this point. The remaining 14 biomarkers followed the expected results (Table 2).

Logistic Regression Models

When clinical variables were included in the regression model, apolipoprotein CIII and NT-proBNP (higher levels in strokes) and FasL (higher levels in stroke mimics) were independent predictors of stroke diagnosis, having this model a predictive accuracy of 0.793 (0.738–0.849) when combined with clinical variables (Table 3, model 1). On ischemic versus hemorrhagic stroke, levels of apolipoprotein CIII, NT-proBNP, Hsc70 (higher in ischemic strokes), and growth-related oncogene-α (higher in hemorrhagic strokes) were independently associated with subtype differentiation together with clinical variables (Table 3, model 2). Accuracy of this model was 0.839 (0.691–0.823).

Table 1. Baseline Characteristics of the Stroke-Chip Cohort and Comparison Between the Different Conditions: Real Strokes Versus Stroke Mimics and Ischemic Versus Hemorrhagic Stroke

	All Patients	Real Strokes	Stroke Mimics	*P* Value	Ischemic Stroke	Hemorrhagic Stroke	*P* Value
Sample size	1308	1115	193	…	941	174	…
Age	73 (61–82)	75 (64–82)	64 (50–75)	<0.0001*	76 (65–83)	70 (59–78)	<0.0001*
Sex (% female)	591 (45.2)	488 (43.8)	103 (53.4)	0.013*	434 (46.1)	54 (31)	<0.0001*
Tobacco	226 (17.3)	186 (16.7)	40 (20.7)	0.170	153 (16.2)	34 (19.5)	0.271
Alcohol	87 (6.7)	81 (7.3)	6 (3.1)	0.032*	65 (6.9)	16 (9.2)	0.285
Arterial hypertension	941 (71.9)	831 (74.5)	110 (57)	<0.0001*	690 (73.3)	141 (81)	0.032*
Diabetes mellitus	329 (25.2)	282 (25.3)	47 (24.4)	0.781	240 (25.5)	42 (24.1)	0.703
Dyslipidemia	616 (47.1)	545 (48.9)	71 (36.8)	0.002*	462 (49.1)	83 (47.7)	0.735
Atrial fibrillation	378 (28.9)	351 (31.5)	27 (14)	<0.0001*	330 (35.1)	21 (12.1)	<0.0001*
Coronary artery disease	185 (14.1)	165 (14.8)	20 (10.4)	0.103	152 (16.2)	13 (7.5)	0.003*
Previous Stroke	237 (18.1)	188 (16.9)	49 (25.4)	0.005*	163 (17.3)	25 (14.4)	0.339
Prestroke mRS score of >2	188 (14.4)	25 (13)	163 (14.6)	0.625	141 (15)	22 (12.6)	0.421
SBP, mm Hg	155 (137–176)	157 (139–178)	144 (127–160)	<0.0001*	155 (136–175)	174 (151–195)	<0.0001*
DBP, mm Hg	84 (73–94)	84 (74–95)	81 (71–92)	0.070*	82 (72–92)	92 (80–104)	<0.0001*
Glycemia, mg/dL	121 (104–148)	122 (106–149)	114 (97–143)	0.001*	121 (104–146)	128 (110–164)	<0.0001*
NIHSS score	6 (3–15)	8 (3–16)	2 (1–6)	<0.0001*	7 (3–15)	12 (6–19)	<0.0001*
OTB time, hh:mm	2:30 (1:40–3:40)	2:30 (1:38–3:40)	2:41 (1:50–3:45)	0.164	2:30 (1:37–3:40)	2:26 (1:40–3:35)	0.819

Data are presented as n (%) for categorical variables and as median (interquartile range) for continuous variables. DBP indicates diastolic blood pressure; mRS, modified Rankin Scale; NIHSS, National Institutes of Health Stroke Scale; OTB time, onset to blood collection time; and SBP, systolic blood pressure.

*P value <0.05.

Table 2. Interim Analysis, Comparison of the Biomarker Values Between the Different Conditions

	All	All Strokes	Stroke Mimics	*P* Value	Ischemic Stroke	Hemorrhagic Stroke	*P* Value
Sample size	541	463	78	…	389	74	…
NT-proBNP	5.80±1.55	5.85±1.46	5.15±1.73	0.001*	5.95±1.45	5.35±1.42	0.001*
IGFBP-3 (std. $\times10^{-5}$)	1.70 (1.56 to 1.98)	1.70 (1.56 to 1.98)	1.68 (1.56 to 4.41)	0.954	1.71 (1.55 to 1.98)	1.69 (1.59 to 1.92)	0.444
TNF-R1 (std. $\times10^{-3}$)	7.1 (6.4 to 7.7)	7.31 (6.4 to 7.8)	6.9 (6.3 to 7.5)	0.113*	7.1 (6.4 to 7.6)	7.1 (6.2 to 7.8)	0.651
GroA	3.45±0.75	3.42±0.72	3.64±0.80	0.014*	3.43±0.73	3.40±0.70	0.738
FasL (std. $\times10^{-2}$)	8.66±2.19	8.54±2.22	8.90±2.22	0.192*	8.50±2.20	8.71±2.34	0.480
IL-6	2.63 (2.08 to 3.27)	2.68 (2.21 to 3.27)	2.32 (1.60 to 2.39)	0.143*	2.63 (2.19 to 3.21)	3.03 (2.32 to 3.71)	0.064*
D-dimer	6.61 (5.98 to 7.39)	6.72 (6.07 to 7.46)	6.25 (5.55 to 6.91)	<0.0001*	6.81 (6.10 to 7.46)	6.52 (5.92 to 7.32)	0.152*
vWF	5.27 (4.75 to 5.68)	5.26 (4.75 to 5.65)	5.23 (4.58 to 5.69)	0.848	5.26 (4.74 to 5.65)	5.34 (4.84 to 5.63)	0.745
VAP-1	12.8 (12.5 to 13.2)	12.8 (12.5 to 13.2)	12.7 (12.4 to 12.9)	0.005*	12.9 (12.6 to 13.3)	12.7 (12.5 to 13.2)	0.563
Endostatin	4.93 (4.65 to 5.19)	4.98 (4.68 to 5.24)	4.74 (4.45 to 4.99)	0.005*	5.00 (4.70 to 5.26)	4.91 (4.54 to 5.12)	0.001*
S100B (std. $\times10^{-2}$)	4.05 (2.67 to 5.13)	3.98 (2.63 to 5.18)	4.06 (2.54 to 4.70)	0.068*	3.85 (2.62 to 5.18)	4.26 (2.84 to 5.22)	0.970
Hsc70 (std.)	0.18 (−0.2 to 0.42)	0.19 (−0.2 to 0.40)	0.14 (−0.3 to 0.49)	0.787	0.25 (−0.1 to 0.42)	−0.03 (−0.4 to 0.37)	0.010*
Apo CIII (std.)	5.63±0.59	5.58±0.62	5.42±0.67	0.029*	5.60±0.59	5.47±0.73	0.150*
NCAM	2.99 (2.68 to 3.85)	2.97 (2.67 to 3.87)	3.18 (2.85 to 4.89)	0.257	2.96 (2.69 to 3.82)	3.0 (2.55 to 4.40)	0.957
MMP-9	11.8 (11.2 to 12.3)	11.8 (11.3 to 12.3)	11.6 (11.2 to 12.3)	0.525	11.8 (11.3 to 12.2)	11.7 (11.3 to 12.3)	0.808
βNGF	−1.59 (−1.59 to −0.50)	−1.59 (−1.59 to −0.50)	−1.59 (−1.59 to −0.58)	0.666	−1.53 (−1.59 to −0.51)	−1.59 (−1.59 to −0.51)	0.778
Caspase-3	1.17 (0.28 to 1.75)	1.19 (0.34 to 1.83)	1.10 (0.60 to 1.70)	0.602	1.18 (0.37 to 4.46)	1.30 (0.29 to 1.85)	0.963
NSE	4.05 (3.71 to 4.32)	4.07 (3.74 to 4.48)	4.04 (3.74 to 4.28)	0.323	4.07 (3.72 to 4.47)	4.09 (3.75 to 4.50)	0.821
cFn	19.5 (19.2 to 19.8)	19.5 (19.2 to 19.8)	19.5 (19.8 to 19.7)	0.599	19.5 (19.2 to 19.8)	19.5 (19.1 to 19.8)	0.811
IL-2RG	−0.22 (−0.68 to 0.24)	−0.16 (−0.62 to 0.23)	−0.22 (−0.55 to 0.11)	0.471	−0.08 (−0.57 to 0.21)	−0.17 (−0.64 to 0.23)	0.748
IL-17A	1.21 (0.92 to 2.05)	1.35 (0.92 to 2.11)	1.48 (0.92 to 2.22)	0.522	1.38 (0.92 to 2.12)	1.17 (0.92 to 2.09)	0.293

This shows mean±SD or median (interquartile range) values of the biomarkers within the different groups of patients. Biomarkers levels are log transformed. In addition, biomarkers disclosing coefficient of variation >20% despite log transformation are standardized by dividing for the internal control in each plate. Apo CIII indicates apolipoprotein CIII; cFn, cellular fibronectin; FasL, Fas ligand; GroA, growth-related oncogene-α; Hsc70, heat shock 70 kDa protein-8; IGFBP-3, insulin-like growth factor-binding protein 3; IL, interleukin; MMP-9, matrix metalloproteinase 9; NCAM, neuron cell adhesion molecule; NSE, neuron-specific enolase; NT-proBNP, N-Terminal Pro-B-Type Natriuretic Peptide; std., standardized values; TNF-R1, tumor necrosis factor receptor-1; VAP-1, vascular adhesion protein-1; vWF, Von Willebrand factor; and βNGF, β-nerve growth factor.

**P* value <0.2.

Validation Cohort

This cohort consisted of 767 patients. There were no significant differences in clinical variables between both cohorts (Table II in the online-only Data Supplement). Seven out of the final 14 biomarkers measured in the whole cohort (IGFBP-3, TNF-R1, FasL, S100B, Hsc70, apolipoprotein CIII, and NCAM) displayed an interassay coefficient of variation >20% between both interim and validation cohort, despite log transformation, and were standardized for the analysis. Accuracy was reduced for both comparisons to 0.742 (0.686–0.797) for strokes versus stroke mimics and to 0.757 (0.691–0.823) for ischemic versus hemorrhagic stroke. Just NT-proBNP remained as an independent predictor for ischemic versus hemorrhagic stroke (Table 4).

An additional analysis of the whole cohort identified NT-proBNP >4.9 (odds ratio, 2.40; 95% confidence interval, 1.55–3.71; *P*<0.0001) and endostatin >4.7 (odds ratio, 2.02; 95% confidence interval, 1.19–3.45; *P*=0.010) as independent predictors of ischemic stroke. The analysis of the whole cohort is detailed in Tables III through V in the online-only Data Supplement and Figures I through III in the online-only Data Supplement.

Discussion

The Stroke-Chip study was designed to test whether a panel of biomarkers could differentiate between real stroke and stroke mimics and between ischemic and hemorrhagic strokes in the acute setting, being to our knowledge the largest biomarker study ever done in the hyperacute phase of stroke. Our results, however, revealed that the studied biomarkers were not sufficient for an accurate differential diagnosis of those entities, despite the validation of some candidates that might be considered for future panels.

Table 3. Interim Analysis, Logistic Regression Models for Both Comparisons

	Odds Ratio	95% Confidence Interval	*P* Value
Model 1: real strokes vs stroke mimics			
Accuracy (AUC): 0.793 (0.738–0.849)			
Apo CIII>5.01	1.89	1.10–3.27	0.022
NT-proBNP>3.47	2.68	1.23–5.86	0.014
FasL>0.11	0.489	0.238–1.004	0.051
Age	1.03	1.01–1.05	0.001
SBP	1.01	1.001–1.02	0.035
Baseline NIHSS	1.14	1.08–1.20	<0.0001
Previous stroke	0.41	0.23–0.76	0.004
Model 2: ischemic vs hemorrhagic stroke			
Accuracy (AUC): 0.839 (0.691–0.823)			
Apo CIII>5.19	2.40	1.19–4.74	0.015
GroA>3.25	0.34	0.16–0.70	0.004
NT-proBNP>5.70	2.30	1.13–4.67	0.021
Hsc70>0.11	3.76	1.86–7.58	<0.0001
Sex	3.07	1.49–6.32	0.002
AF	4.22	1.77–10.08	0.001
SBP	0.98	0.97–0.99	<0.0001
Baseline NIHSS	0.90	0.87–0.94	<0.0001

This shows the final models of the logistic regression analyses for the comparison between real strokes vs stroke mimics and between ischemic and hemorrhagic strokes, after including in first step all variables associated with the dependent variable at a *P* value >0.1. Odds ratios are referred to real strokes (model 1) and ischemic stroke (model 2). AF indicates atrial fibrillation; Apo CIII, apolipoprotein CIII; AUC, area under the curve; FasL, Fas ligand; GroA, growth-related oncogene-α; Hsc70, heat shock 70 kDa protein-8; NIHSS, National Institutes of Health Stroke Scale; NT-proBNP, N-Terminal Pro-B-Type Natriuretic Peptide; and SBP, systolic blood pressure.

Table 4. Validation Cohort, Logistic Regression Models for Both Comparisons

	Odds Ratio	95% Confidence Interval	*P* Value
Model 1: real strokes vs stroke mimics			
Accuracy (AUC): 0.742 (0.686–0.797)			
Apo CIII>5.01	1.10	0.61–1.95	0.777
NT-proBNP>3.47	1.12	0.39–3.18	0.839
FasL>0.11	0.71	0.39–1.26	0.241
Age	1.03	1.01–1.05	0.001
SBP	1.02	1.01–1.03	<0.0001
Baseline NIHSS	1.10	1.05–1.15	<0.0001
Previous stroke	0.38	0.20–0.72	0.003
Model 2: ischemic vs hemorrhagic stroke			
Accuracy (AUC): 0.757 (0.691–0.823)			
Apo CIII>5.19	0.63	0.291.37	0.243
GroA>3.25	0.89	0.46–1.75	0.743
NT-proBNP>5.70	2.85	1.48–5.51	0.002
Hsc70>0.11	3.85	0.66–22.32	0.133
Sex	1.68	0.90–3.14	0.101
AF	2.41	1.01–5.76	0.047
SBP	0.98	0.97–0.99	<0.0001
Baseline NIHSS	0.93	0.89–0.96	<0.0001

This shows the validation of the logistic regression models obtained from the interim analysis for the comparison between real strokes vs stroke mimics and between ischemic and hemorrhagic strokes. Odds ratios are referred to real strokes (model 1) and ischemic stroke (model 2). AF indicates atrial fibrillation; AUC, area under the curve; FasL, Fas ligand; GroA, growth-related oncogene-α; Hsc70, heat shock 70 kDa protein-8; NIHSS, National Institutes of Health Stroke Scale; NT-proBNP, N-Terminal Pro-B-Type Natriuretic Peptide; and SBP, systolic blood pressure.

The best biomarkers indentified in our study represent important steps in pathophysiological pathways involved in stroke, such as cardiac diseases (NT-proBNP), angiogenesis (endostatin) coagulation, and hemostasis (D-dimer). In fact, none of them are a brain-specific biomarker, which is reflected by higher levels in ischemic stroke rather than hemorrhagic stroke patients, in whom more and faster tissue destruction is expected; therefore, incorporation of brain-specific biomarkers, such as GFAP (glial fibrillary acid protein), would be more useful for intracerebral hemorrhage recognition and inclusion into this panel might result in better accuracies. Surprisingly, the identified biomarkers, with the exception of D-dimer,[6] were previously identified not as diagnostic biomarkers, but as markers of poor prognosis[13,18] or cardioembolic origin.[19] Therefore, it seems reasonable that the better accuracy could be obtained by a panel combining markers from the different pathways involved in stroke.

Stroke mimics usually represent benign conditions that, even requiring medical attention,[4] do not really need an emergent transfer or admission to stroke units. A recent study has shown that >20% of patients with suspected stroke that are transferred by helicopter are finally stroke mimics.[20] A high-sensitivity test able to rule out stroke diagnosis would avoid that. In comparison with previous studies, we found a relatively low rate of stroke mimics (around 15%), probably because of the fact of enrolling patients at hospital arrival by neurologists. These percentages might have an influence on the overall study results, underestimating the value of the biomarkers in differentiating real strokes and stroke mimics. Future studies should explore the same issue at the prehospital level, where the rate of stroke mimics should be higher. In any case, the obtained results in our study for differentiating stroke mimics from real strokes were not optimal. However, when comparing them with imaging tests in such scenario, we find that simple computed tomography scans, despite a high specificity close to 100% to exclude hemorrhagic stroke, have just modest sensitivity (around 30%) for ischemic stroke diagnosis in the acute phase.[21]

On the differentiation between ischemic and hemorrhagic strokes, the biomarker panel proposed should raise, at least, specificity rates close to 100% to safely discard the possibility to give tPA to a patient with an intracerebral hemorrhage.

In our study, the best model accuracy was >80%. However, to be applied in clinical practice, accuracy of the model must be improved. The addition of new blood biomarkers, such as GFAP and retinol-binding protein 4 (RBP4),[22] might result in a more accurate model.

Our results represent to date the best results in stroke diagnosis by blood biomarkers for a large cohort. In a previous study from our group, the best accuracy reached by a 6-biomarker panel was 75.9%.[6] A previous study with similar sample size testing a 4-biomarker panel (MMP-9, BNP, D-dimer, and S100B) disclosed accuracies of 76% for hemorrhagic stroke and 69% for all stroke.[14] In a recent study, including >500 patients, the neuroendocrine biomarker copeptin was unable to distinguish between strokes and stroke mimics.[23] Unlike the previous studies, the Stroke-Chip has used a mixed approach to develop diagnostic models, based on clinical variables plus biomarker information, a strategy that has been able to improve its accuracy rates. However, these numbers could be useful at some level in less developed countries, where not all patients are able to receive a neuroimaging test.

The prospective design, the large sample size, and the fact that the candidate biomarkers are based on our own previous results and the literature represent the main strengths of the Stroke-Chip study. However, the study has also some limitations. Perhaps, the main limitation was the low reproducibility between the interim and the final analysis. The need to standardize the results could lead to an underestimation of the biomarker effect. In fact, no one of the standardized biomarkers revealed significant differences between the compared subgroups. This was not the case of the biomarkers finally included into the logistic regression models (IL-6, NT-proBNP, D-dimer), which represent well-known and set-up molecules. Beyond this limitation, other minor issues should be also mentioned, such as the analysis looking for the best cutoffs for accuracy, strategy that is known to overestimate the biomarker effect. Finally, we are aware that the inclusion of patients with conditions potentially influencing some of the investigated circulating biomarker levels, such as infections, inflammatory disorders, renal, or hepatic dysfunction, may have had an impact on the association between circulating biomarker levels and stroke diagnosis. However, we decided to not exclude these patients, as the aim of the study was the development of a diagnostic test that could be applicable to every stroke patient regardless of any medical condition.

In conclusion, the Stroke-Chip study has identified some candidates for the biochemical diagnosis of stroke in the largest study of biomarkers conducted in the acute phase of stroke to date. However, the accuracy rates of the predictive models, even after combining with clinical variables, are still far from those requested in a real scenario. Discovery of new biomarkers and improvement on laboratory techniques remain the next challenges to achieve the molecular diagnosis of stroke.

Acknowledgments

We want to acknowledge all the neurologists and nurses from the recruiting centers, for their kind support in the study.

Sources of Funding

The Stroke-Chip study was supported by the research project IDI-20130009, Diseño, desarrollo y validación de una herramienta Laboratory-on-a-chip para el diagnóstico precoz del ictus y su diferenciación de patologías análogas, from the Centro para el Desarrollo Tecnológico Industrial (CDTI). Neurovascular Research Laboratory from Vall d'Hebron Institute of Research and Neuroscience Unit from Institut d'Investigació Germans Trias i Pujol take part into the Spanish stroke research network INVICTUS+ (RD16/0019/0021). Neurovascular Research Laboratory is supported in stroke biomarkers research by Fondo de Investigaciones Sanitarias PI15/354, cofinanced by the European Regional Development Fund (FEDER). The study founders had no role in the study design and conduction.

Disclosures

Dr Bustamante is supported by a Juan Rodés contract JR16/00008 from the Instituto de Salud Carlos III. Dr López-Cancio is supported by a Juan Rodés contract JR14/00041 from the Instituto de Salud Carlos III. Drs Gasull and Dávalos are inventors of the patent WO 2012152970 A1, which includes the biomarker Hsc70. Dr Montaner is an inventor of the European patents with number EP20090811142, which include the biomarker VAP-1, and EP2492691A1, which include the biomarker endostatin. The other authors report no conflicts.

References

1. Benjamin EJ, Blaha MJ, Chiuve SE, Cushman M, Das SR, Deo R, et al; American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart Disease and Stroke Statistics-2017 Update: a report from the American Heart Association. *Circulation*. 2017;135:e146–e603. doi: 10.1161/CIR.0000000000000485.
2. Lees KR, Bluhmki E, von Kummer R, Brott TG, Toni D, Grotta JC, et al; ECASS, ATLANTIS, NINDS and EPITHET rt-PA Study Group. Time to treatment with intravenous alteplase and outcome in stroke: an updated pooled analysis of ECASS, ATLANTIS, NINDS, and EPITHET trials. *Lancet*. 2010;375:1695–1703. doi: 10.1016/S0140-6736(10)60491-6.
3. Ebinger M, Winter B, Wendt M, Weber JE, Waldschmidt C, Rozanski M, et al; STEMO Consortium. Effect of the use of ambulance-based thrombolysis on time to thrombolysis in acute ischemic stroke: a randomized clinical trial. *JAMA*. 2014;311:1622–1631. doi: 10.1001/jama.2014.2850.
4. Hand PJ, Kwan J, Lindley RI, Dennis MS, Wardlaw JM. Distinguishing between stroke and mimic at the bedside: the brain attack study. *Stroke*. 2006;37:769–775. doi: 10.1161/01.STR.0000204041.13466.4c.
5. Tsivgoulis G, Zand R, Katsanos AH, Goyal N, Uchino K, Chang J, et al. Safety of intravenous thrombolysis in stroke mimics: prospective 5-year study and comprehensive meta-analysis. *Stroke*. 2015;46:1281–1287. doi: 10.1161/STROKEAHA.115.009012.
6. Montaner J, Mendioroz M, Ribó M, Delgado P, Quintana M, Penalba A, et al. A panel of biomarkers including caspase-3 and D-dimer may differentiate acute stroke from stroke-mimicking conditions in the emergency department. *J Intern Med*. 2011;270:166–174. doi: 10.1111/j.1365-2796.2010.02329.x.
7. Hatano S. Experience from a multicentre stroke register: a preliminary report. *Bull World Health Organ*. 1976;54:541–553.
8. Brott T, Adams HP Jr, Olinger CP, Marler JR, Barsan WG, Biller J, et al. Measurements of acute cerebral infarction: a clinical examination scale. *Stroke*. 1989;20:864–870.
9. Bustamante A, Sobrino T, Giralt D, García-Berrocoso T, Llombart V, Ugarriza I, et al. Prognostic value of blood interleukin-6 in the prediction of functional outcome after stroke: a systematic review and meta-analysis. *J Neuroimmunol*. 2014;274:215–224. doi: 10.1016/j.jneuroim.2014.07.015.
10. Castellanos M, Leira R, Serena J, Blanco M, Pedraza S, Castillo J, et al. Plasma cellular-fibronectin concentration predicts hemorrhagic transformation after thrombolytic therapy in acute ischemic stroke. *Stroke*. 2004;35:1671–1676. doi: 10.1161/01.STR.0000131656.47979.39.
11. De Meyer SF, Stoll G, Wagner DD, Kleinschnitz C. von Willebrand factor: an emerging target in stroke therapy. *Stroke*. 2012;43:599–606. doi: 10.1161/STROKEAHA.111.628867.

12. Hernandez-Guillamon M, Garcia-Bonilla L, Solé M, Sosti V, Parés M, Campos M, et al. Plasma VAP-1/SSAO activity predicts intracranial hemorrhages and adverse neurological outcome after tissue plasminogen activator treatment in stroke. *Stroke*. 2010;41:1528–1535. doi: 10.1161/STROKEAHA.110.584623.
13. Navarro-Sobrino M, Rosell A, Hernández-Guillamon M, Penalba A, Boada C, Domingues-Montanari S, et al. A large screening of angiogenesis biomarkers and their association with neurological outcome after ischemic stroke. *Atherosclerosis*. 2011;216:205–211. doi: 10.1016/j.atherosclerosis.2011.01.030.
14. Laskowitz DT, Kasner SE, Saver J, Remmel KS, Jauch EC. Clinical usefulness of a biomarker-based diagnostic test for acute stroke: the Biomarker Rapid Assessment in Ischemic Injury (BRAIN) study. *Stroke*. 2009;40:77–85.
15. Allard L, Lescuyer P, Burgess J, Leung KY, Ward M, Walter N, et al. ApoC-I and ApoC-III as potential plasmatic markers to distinguish between ischemic and hemorrhagic stroke. *Proteomics*. 2004;4:2242–2251.
16. Undén J, Strandberg K, Malm J, Campbell E, Rosengren L, Stenflo J, et al. Explorative investigation of biomarkers of brain damage and coagulation system activation in clinical stroke differentiation. *J Neurol*. 2009;256:72–77.
17. Hayashi T, Seki T, Sato K, Iwai M, Zhang WR, Manabe Y, et al. Expression of polysialylated neural cell adhesion molecule in rat brain after transient middle cerebral artery occlusion. *Brain Res*. 2001;907:130–133.
18. García-Berrocoso T, Giralt D, Bustamante A, Etgen T, Jensen JK, Sharma JC, et al. B-type natriuretic peptides and mortality after stroke: a systematic review and meta-analysis. *Neurology*. 2013;81:1976–1985. doi: 10.1212/01.wnl.0000436937.32410.32.
19. Llombart V, Antolin-Fontes A, Bustamante A, Giralt D, Rost NS, Furie K, et al. B-type natriuretic peptides help in cardioembolic stroke diagnosis: pooled data meta-analysis. *Stroke*. 2015;46:1187–1195. doi: 10.1161/STROKEAHA.114.008311.
20. Sequeira D, Martin-Gill C, Kesinger MR, Thompson LR, Jovin TG, Massaro LM, et al. Characterizing strokes and stroke mimics transported by helicopter emergency medical services. *Prehosp Emerg Care*. 2016;20:723–728. doi: 10.3109/10903127.2016.1168889.
21. Patel SC, Levine SR, Tilley BC, Grotta JC, Lu M, Frankel M, et al; National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. Lack of clinical significance of early ischemic changes on computed tomography in acute stroke. *JAMA*. 2001;286:2830–2838. doi: 10.1001/jama.286.22.2830.
22. Llombart V, García-Berrocoso T, Bustamante A, Giralt D, Rodriguez-Luna D, Muchada M, et al. Plasmatic retinol-binding protein 4 and glial fibrillary acidic protein as biomarkers to differentiate ischemic stroke and intracerebral hemorrhage. *J Neurochem*. 2016;136:416–424. doi: 10.1111/jnc.13419.
23. Wendt M, Ebinger M, Kunz A, Rozanski M, Waldschmidt C, Weber JE, et al; STEMO Consortium. Copeptin levels in patients with acute ischemic stroke and stroke mimics. *Stroke*. 2015;46:2426–2431. doi: 10.1161/STROKEAHA.115.009877.

Blood Biomarkers for the Early Diagnosis of Stroke: The Stroke-Chip Study

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Stroke. 2017;48:2419-2425; originally published online July 17, 2017;
doi: 10.1161/STROKEAHA.117.017076
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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

ONLINE SUPPLEMENT

BLOOD BIOMARKERS FOR THE EARLY DIAGNOSIS OF STROKE. THE STROKE-CHIP STUDY

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SUPPLEMENTAL METHODS I: DISCOVERY PHASE: STROKE VS. STROKE-MIMICS PANEL

Methods

Patients for this phase were recruited in the emergency department of Vall d'Hebron Hospital as previously published [1]. Briefly, stroke suspicions of less than 24 hours from symptoms onset were enrolled. Blood samples were obtained at patient's arrival, and later a complete diagnostic protocol allowed deciding the final diagnosis of stroke or mimicking condition. A population of 230 cases was analyzed in the present study. Of them, 146 were stroke patients, 61 stroke-mimics and 23 healthy controls. Subjects of these groups were randomly selected from our sample bank in order to select an approximate 30% rate of stroke-mimics.

Each plasma sample was assayed in duplicate using an antibody-based array of SearchLight Technology (Aushon BioSystems, Billerica, MA, USA) and mean value of both measurements was used. After screening of 125 proteins in a small group of selected and well phenotyped stroke patients (N=9), stroke-mimics (N=2) and healthy controls (N=4), 22 key biomarkers were selected and combined in customs arrays supplied by Aushon BioSystems. Proteins of interest were distributed in 9 different panel arrays: *array 1*: interleukin-17 (IL-17A), myeloid progenitor inhibitory factor 1 (MPIF-1), interleukin-2 receptor gamma (IL-2RG) and Fas ligand (FasL); *array 2*: cluster of differentiation-14 (CD14); *array 3*: osteopontin (OPN); *array 4*: beta nerve growth factor (bNGF); *array 5*: interleukin-16 (IL-16), interleukin-1 receptor alpha (IL-1RA), interferon gamma (IFN-gamma), granulocyte colony-stimulating factor (G-CSF), leptin, human growth hormone (HGH), interferon gamma-induced protein 10 (IP-10); *array 6*: insulin-like growth factor binding protein 3 (IGFBP-3), insulin-like growth factor binding protein 1 (IGFBP-1), tumor necrosis factor receptor 1 (TNF-R1), growth-related oncogen alpha (GroA), immunoglobulin E (IgE); *array 7*: E-cadherin; *array 8*: granulocyte macrophage colony-stimulating factor (GM-CSF); and *array 9*: platelet-derived growth factor BB (PDGF-BB). All these proteins showed statistical significance ($p<0.05$) to distinguish stroke from stroke mimicking conditions, and therefore were tested in all 230 included subjects.

Results

From the screening in an antibody-based array, it was possible to identify 23 protein biomarkers useful to differentiate stroke from mimics. After selecting the best cut-off level for each biomarker looking for a compromise between better sensitivity offering acceptable specificity, six biomarkers were chosen, as being independent predictors in a logistic regression model including at the first step all 23 biomarkers. Three of those markers were significantly elevated in stroke patients (IL-17A, bNGF and FasL) while the other three were lower among stroke patients compared with stroke-mimics (IGFBP-3, TNF-R1 and GroA). Several clinical variables such as hypertension, atrial fibrillation, dyslipidemia and tobacco were also independent predictors of stroke diagnosis. The final model integrating clinical variables and biomarkers data is shown in the next table.

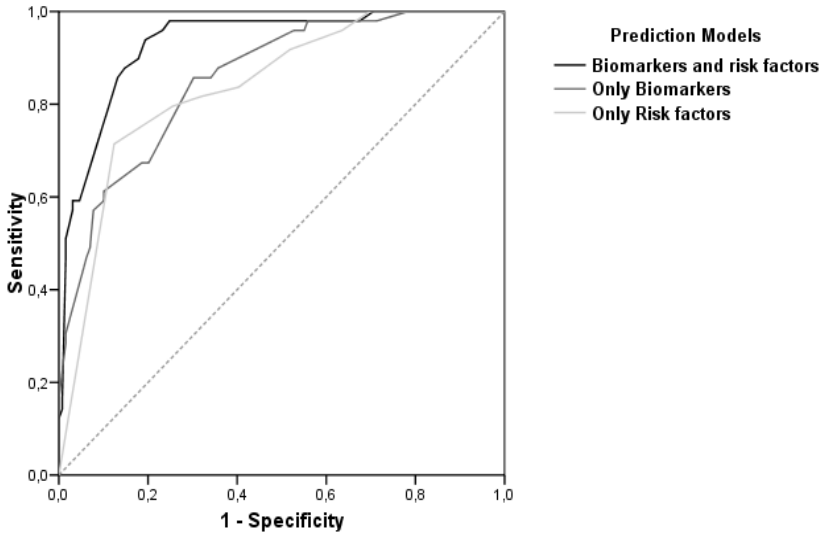
Logistic regression analysis

	Odds ratio (stroke)	95% confidence interval	p
Clinical Factors			
Hypertension	3.711	1.554-8.862	0.003
Atrial Fibrillation	19.661	4.367-88.524	<0.001
Dyslipidemia	3.946	1.303-11.946	0.015
Tobacco	7.586	2.041-28.192	0.002
Biomarkers			
IL-17 >0.05	31.833	2.297-441.175	0.01
FasL >13.8	2.847	1.287-6.298	0.01
bNGF>1.25	7.843	2.80-21.973	<0.001
IGFBP-3 <2165872.35	8.954	1.649-48.629	0.011
TNF-R1 <3271	3.250	1.474-7.168	0.003
GroA <424.6	2.619	1.023-6.705	0.045
Clinical Factors + Biomarkers			
IL-17 >0.05	114.766	3.496-3767.611	0.008
bNGF >1.25	9.193	2.264-37.323	0.002
IGFBP-3 <2165872.35	34.552	2.613-456.953	0.007
TNF-R1 <3291	8.699	2.877-26.299	<0.001
Hypertension	3.467	1.125-10.687	0.03
Atrial Fibrillation	26.72	4.766-149.805	<0.001
Dyslipidemia	8.116	1.795-36.697	0.007

The table represents the final results of the logistic regression analysis for 3 different predictive models, the first one including just clinical variables, a second one including just blood biomarkers, and the final model including both clinical factors and biomarkers. IL-17A: interleukin-17A; FasL: Fas ligand; bNGF: beta nerve growth factor; IGFBP-3: insulin-like growth factor binding protein 3; TNF-R1: tumor necrosis factor receptor 1; GroA: growth-related oncogen alpha.

Receptor operating characteristics (ROC) curves were constructed comparing the accuracy of the selected biomarkers (IL-17A, FasL, bNGF, GroA, IGFBP-3 and TNF-R1), clinical variables (hypertension, atrial fibrillation, dyslipidemia or tobacco) or the combination of biomarkers and clinical variables (IL-17, bNGF, IGFBP-3, TNF-R1, hypertension, atrial fibrillation and dyslipidemia) to distinguish between strokes and stroke-mimics. Although the biomarkers model had better area under de curve (AUC=0.856) than the clinical model (AUC=0.833), the model combining biomarkers and clinical variables offered the best AUC (0.933), adding significant information to both clinical data alone and biomarkers alone (as seen in the figure).

Accuracy of the predictive models for stroke vs. stroke-mimics differentiation



SUPPLEMENTAL METHODS II: METHODS OF BIOMARKER MEASUREMENT, MATERIAL, DILUTION AND UNITS

Biomarker	Manufacturer	Reference	Dilution	LLOQ	Units
IL-17A	Diaclone	850,940,192	1/2	2.3	pg/mL
IL-2RG	Cusabio	CSB-EL011651Hu	1/2	0.078	ng/mL
NT-proBNP	Roche	4,842,464	1/1	5	pg/mL
IGFBP-3, TNF-R1	Aushon 2-plex	85,214	1/25	48.8, 2.34	pg/mL
GroA, FasL, IL-6	Aushon 3-plex	85,723	1/2	0.39, 1.56, 0.20	pg/mL
cFn	Aushon 1-plex	85,725	1/50,000	117.2	pg/mL
D-dimer	Stago	947	1/42	10	ng/mL
vWF	Stago	942	1/102	1	%
MMP-9	Aushon 1-plex	84,920	1/100	48.8	pg/mL
VAP-1	eBioscience	BMS259TEN	1/1000	0.019	ng/mL
Endostatin	R&D	RYD-DNST0	1/50	0.023	ng/mL
Caspase-3	eBioscience	BMS2012INTS	1/3	0.12	ng/mL
bNGF	Aushon 1-plex	84,990	1/1,25	0.39	pg/mL
Hsc70	USCNK/Cloud-Clone Corp	E93063Hu/ SED063Hu	1/1	0.134	ng/mL
S100B	Cusabio	CSB-E08065h	1/1	1.17	pg/mL
NCAM	Abnova	KA2003	1/150	10	pg/mL
NSE	Abnova	KA2122	1/10	0.15	ng/mL
Apo-CIII	Abnova	KA0465	1/2500	0.002	µg/mL

LLOQ: lower limit of quantification; IL-17A: interleukin-17A; IL-2RG: interleukin-2 receptor gamma; NT-proBNP: N-terminal pro B-type natriuretic peptide; IGFBP-3: insulin-like growth factor-binding protein 3; TNF-R1: tumor necrosis factor receptor 1; GroA: growth-related oncogen alpha; FasL: Fas ligand; IL-6: interleukin-6; cFn: cellular fibronectin; vWF: Von Willebrand factor; MMP-9: matrix metalloproteinase 9; VAP-1: vascular adhesion protein-1; bNGF: beta nerve growth factor; Hsc70: heat shock 70 kDa protein 8; NCAM: neuron cell adhesion molecule; NSE: neuron-specific enolase.

SUPPLEMENTAL TABLE I: BASELINE CHARACTERISTICS OF THE INTERIM ANALYSIS COHORT AND COMPARISON BETWEEN THE DIFFERENT CONDITIONS: REAL STROKES vs. STROKE-MIMICS AND ISCHEMIC vs. HEMORRHAGIC STROKE

	ALL	ALL STROKES	STROKE-MIMICS	P	ISCHEMIC STROKE	ICH	P
Sample size	541	463	78	-	389	74	-
Age	75 (63-82)	76 (65-82)	64.5 (50-75)	<0.0001*	76 (65-82)	73.5 (64-79)	0.011*
Sex (% female)	245 (45.3%)	204 (44.1%)	41 (52.6%)	0.163	182 (46.8%)	22 (29.7%)	0.007*
Tobacco	94 (17.4%)	77 (16.6%)	17 (21.8%)	0.265	57 (14.7%)	20 (27%)	0.009*
Alcohol	35 (6.5%)	32 (6.9%)	3 (3.8%)	0.309	21 (5.4%)	11 (14.9%)	0.003*
Arterial hypertension	400 (73.9%)	355 (76.7%)	45 (57.7%)	<0.0001*	294 (75.6%)	61 (82.4%)	0.201
Diabetes mellitus	144 (26.6%)	127 (27.4%)	17 (21.8%)	0.298	106 (27.2%)	21 (28.4%)	0.842
Dyslipidemia	250 (16.2%)	225 (48.6%)	25 (32.1%)	0.007*	188 (48.3%)	37 (50%)	0.792
Atrial fibrillation	169 (31.2%)	156 (33.7%)	13 (16.7%)	0.003*	145 (37.3%)	11 (14.9%)	<0.0001*
Coronary artery disease	84 (15.5%)	74 (16%)	10 (12.8%)	0.476	63 (16.2%)	11 (14.9%)	0.775
Previous Stroke	111 (20.5%)	92 (19.9%)	19 (24.4%)	0.364	81 (20.8%)	11 (14.9%)	0.239
Pre-stroke mRS>2	88 (16.3%)	75 (16.2%)	13 (16.7%)	0.787	66 (17%)	9 (12.2%)	0.314
SBP (mmHg)	160 (140-179.5)	161 (141-180)	148 (132-171)	0.007*	160 (140-177)	174.5 (156-196)	<0.0001*
DBP (mmHg)	84 (74-96)	85 (75-96)	81 (71-94)	0.137	84 (73-94)	92 (83.5-100)	<0.0001*
Glycemia (mg/dL)	123 (105-155.5)	124 (106-157)	117.5 (95-139)	0.039*	122 (104-151)	134 (116-186.5)	0.001*
NIHSS score	7 (3-15)	8 (3-16)	2 (1-6)	<0.0001*	7 (3-15)	13.5 (6-20)	<0.0001*

ICH: intracerebral hemorrhage (hemorrhagic stroke); mRS: modified Rankin scale; SBP: systolic blood pressure; DBP: diastolic blood pressure; NIHSS: national institutes of health stroke scale. * denotes p value <0.05, and these variables were included at the first step to develop logistic regression models.

SUPPLEMENTAL TABLE II: DESCRIPTIVE DATA AND COMPARISON BETWEEN THE INTERIM AND VALIDATION COHORTS

	All	Interim	Validation	p
Sample size	1,308	541	767	-
Stroke-mimics	193(14.8)	78(14.4)	115(15)	0.772
Hemorrhagic stroke	174(13.3)	74(13.7)	100(13)	0.737
Age	73(61-82)	75(63-82)	73(60.5-82)	0.408
Sex (% female)	591(45.2)	245(45.3)	346(45.1)	0.950
Tobacco	226(17.3)	94(17.4)	132(17.2)	0.938
Alcohol	87(6.7)	35(6.5)	52(6.8)	0.825
Arterial hypertension	941(71.9)	400(73.9)	541(70.5)	0.177
Diabetes mellitus	329(25.2)	144(26.6)	185(24.1)	0.305
Dyslipidemia	616(47.1)	250(46.2)	366(47.7)	0.591
Atrial fibrillation	378(28.9)	169(31.2)	209(27.2)	0.117
Coronary artery disease	185(14.1)	84(15.5)	101(13.2)	0.228
Previous Stroke	237(18.1)	111(20.5)	126(16.4)	0.059
Pre-stroke mRS>2	188(14.4)	88(17.2)	100(13.2)	0.053
NIHSS score	6(3-15)	7(3-15)	6(2-15)	0.582

The table represents the comparison of baseline variables between the interim and validation cohorts. Data are expressed as N (%) or median (interquartile range), as appropriate. mRS: modified Rankin scale; NIHSS: National Institutes of Health Stroke Scale.

SUPPLEMENTAL TABLE III: COMPARISON OF BIOMARKER VALUES BETWEEN THE DIFFERENT CONDITIONS IN THE WHOLE COHORT

	ALL	ALL STROKES	STROKE-MIMICS	P	ISCHEMIC STROKE	HEMORRHAGIC STROKE	P
Sample size	1,308	1,115	193	-	941	174	-
NT-proBNP	5.7 ± 1.5	5.8 ± 1.5	5.1 ± 1.6	<0.0001*	5.9 ± 1.5	5.2 ± 1.4	<0.0001*
IGFBP-3 (std. x10^{-5})	1.8 (1.6-2.1)	1.8 (1.6-2.1)	1.8 (1.6-2.1)	0.888	1.8 (1.6-2.1)	1.8 (1.6-2.1)	0.651
GroA	3.6 ± 0.7	3.6 ± 0.7	3.7 ± 0.8	0.086	3.6 ± 0.7	3.6 ± 0.7	0.682
FasL (std. x10^{-2})	8.8 (7.5-10.3)	8.8 (7.5-10.1)	9.2 (7.5-11.2)	0.144	8.7 (7.5-10.1)	9.2 (7.6-10.6)	0.387
IL-6	2.6 (2.2-3.5)	2.8 (2.3-3.5)	2.4 (1.7-3.2)	<0.0001*	2.8 (2.2-3.4)	3.1 (2.4-3.7)	0.002*
D-dimer	6.7 (6.1-7.4)	6.8 (6.2-7.4)	6.4 (5.7-7.0)	<0.0001*	6.8 (6.2-7.5)	6.5 (6.1-7.3)	0.003*
vWF	4.9 (4.3-5.4)	4.9 (4.3-5.43)	4.9 (4.2-5.4)	0.497	4.8 (4.3-5.4)	5.0 (4.4-5.5)	0.274
VAP-1	12.7 (12.5-13.0)	12.8 (12.5-13.0)	12.6 (12.4-12.9)	<0.0001*	12.8 (12.5-13.0)	12.7 (12.5-13.0)	0.301
Endostatin	5.1 (4.9-5.4)	5.2 (4.9-5.4)	5.0 (4.7-5.5)	<0.0001*	5.2 (5.0-5.5)	5.1 (4.8-5.3)	<0.0001*
Apo-CIII (std.)	5.0 (4.7-5.5)	5.0 (4.7-5.4)	5.0 (4.7-5.5)	0.217	5.0 (4.7-5.4)	5.0 (4.7-5.4)	0.267

*The table shows median (interquartile range) or mean ± standard deviation for log-transformed values of each biomarker in the whole cohort, real strokes vs. stroke-mimics and ischemic vs. hemorrhagic stroke. Seven out of the final 14 biomarkers measured in the whole cohort (IGFBP-3, TNF-R1, FasL, S100B, Hsc70, Apo-CIII, NCAM) displayed an inter-assay CV>20%, and were standardized for the analysis. Even after standardization, significant differences were noted on TNF-R1, NCAM, S100B and Hsc70 between interim and final cohorts and, therefore, could not be analyzed together. Just biomarkers measured in the whole cohort are included. NT-proBNP: N-terminal pro B-type natriuretic peptide; IGFBP-3: insulin-like growth factor-binding protein 3; GroA: growth-related oncogen alpha; FasL: Fas ligand; IL-6: interleukin-6; vWF: Von Willebrand factor; VAP-1: vascular adhesion protein-1. Std: standardized values. * denotes p value <0.05*

SUPPLEMENTAL TABLE IV: LOGISTIC REGRESSION MODELS FOR THE WHOLE COHORT

When all relevant clinical variables were included in the regression model, none of the studied biomarkers was found to be independently associated with the discrimination between stroke and stroke-mimics (model 1). When excluding NIHSS from the model, D-dimer was the only biomarker that independently differentiated those entities (model 2). However, accuracy (AUC) of the model was reduced with this second approach (80.8% in model 1 versus 75.8% in model 2).

A. Real strokes vs. stroke-mimics

	Odds ratio (stroke)	**95% confidence interval**	**p**
MODEL 1: INCLUDING BASELINE NIHSS			
ACCURACY (AUC): 0.808 (0.77-0.85)			
Age	1.03	1.02-1.05	<0.0001
Sex (female)	0.48	0.33-0.70	<0.0001
Alcohol	2.74	1.11-6.75	0.028
Dyslipidemia	1.54	1.05-2.24	0.026
Atrial fibrillation	2.22	1.31-3.75	0.003
Previous stroke	0.33	0.22-0.52	<0.0001
SBP	1.02	1.01-1.02	<0.0001
Baseline NIHSS	1.12	1.08-1.16	<0.0001
MODEL 2: NOT INCLUDING BASELINE NIHSS			
ACCURACY (AUC): 0.758 (0.72-0.80)			
Age	1.03	1.02-1.04	<0.0001
Sex (female)	0.48	0.34-0.69	<0.0001
Alcohol	2.42	0.99-5.91	0.053
Dyslipidemia	1.55	1.08-2.24	0.018
Atrial fibrillation	2.75	1.65-4.58	<0.0001
Previous stroke	0.37	0.24-0.56	<0.0001
D-dimer>6.015 ng/mL	1.79	1.17-2.73	0.007

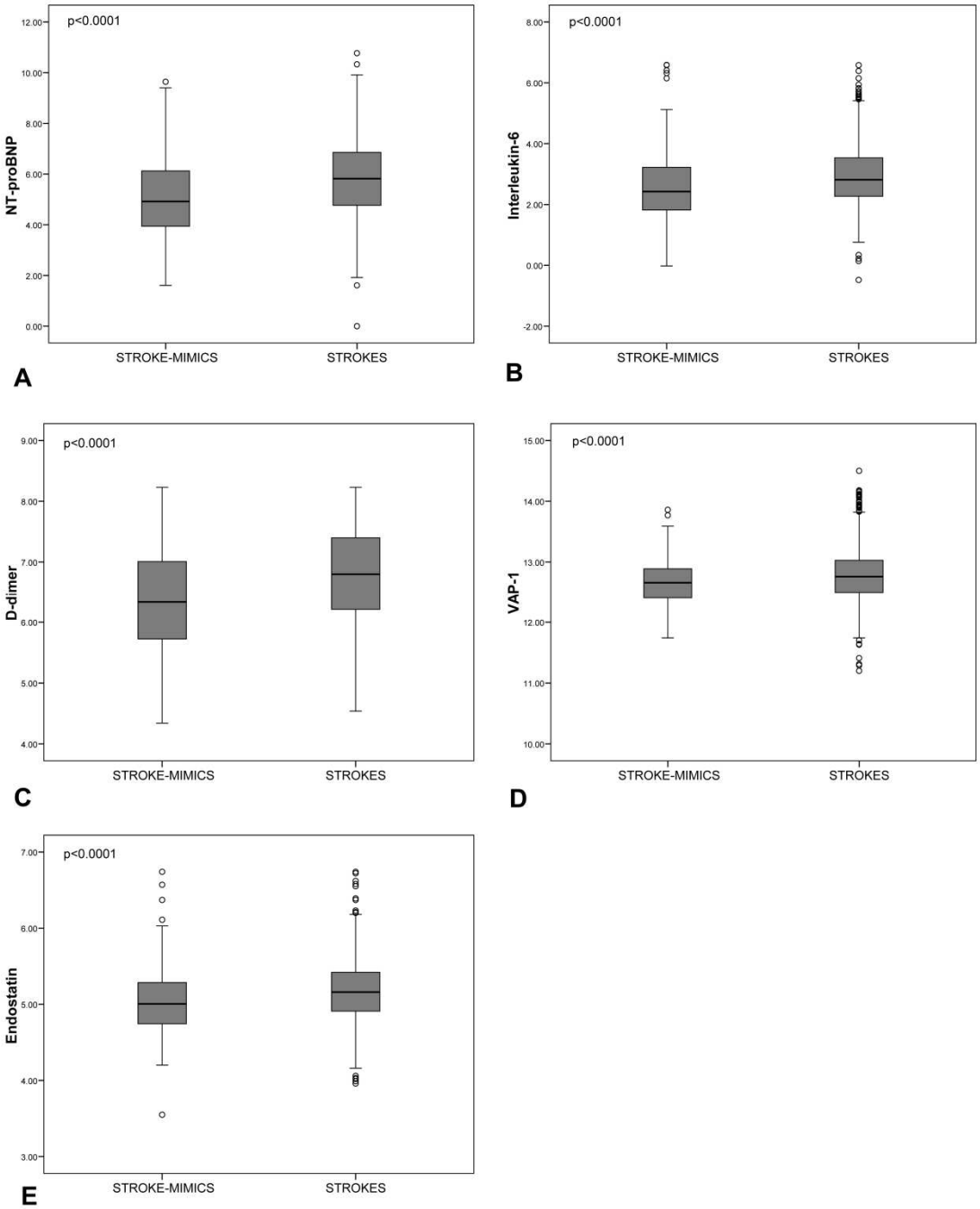
Regarding ischemic vs. hemorrhagic stroke, NT-proBNP and endostatin levels were independently associated with subtype differentiation together with other clinical variables (model 1). Similar results were found after excluding NIHSS (model 2). Accuracy of the model was again reduced (80.6% in model 1 versus 77.7% in model 2).

B. Ischemic vs. hemorrhagic stroke

	Odds ratio (IS)	95% confidence interval	p
MODEL 1: INCLUDING BASELINE NIHSS			
ACCURACY (AUC): 0.806 (0.77-0.85)			
Sex (female)	2.00	1.23-3.08	0.002
Hypertension	0.46	0.28-0.76	0.003
Atrial fibrillation	4.65	2.57-8.42	<0.0001
SBP	0.99	0.98-0.99	0.002
DBP	0.98	0.98-0.99	0.015
Baseline NIHSS	0.91	0.86-0.93	<0.0001
NT-proBNP>4.900 pg/mL	2.40	1.55-3.71	<0.0001
Endostatin>4.675 ng/mL	2.02	1.19-3.45	0.010
MODEL 2: NOT INCLUDING BASELINE NIHSS			
ACCURACY (AUC): 0.777 (0.74-0.82)			
Sex (female)	1.99	1.30-3.04	0.001
Hypertension	0.46	0.28-0.77	0.003
Atrial fibrillation	4.67	2.61-8.35	<0.0001
SBP	0.99	0.98-0.99	0.001
DBP	0.99	0.97-0.99	0.027
NT-proBNP>4.900 pg/mL	2.20	1.44-3.37	<0.0001
Endostatin>4.675 ng/mL	2.16	1.28-3.65	0.004

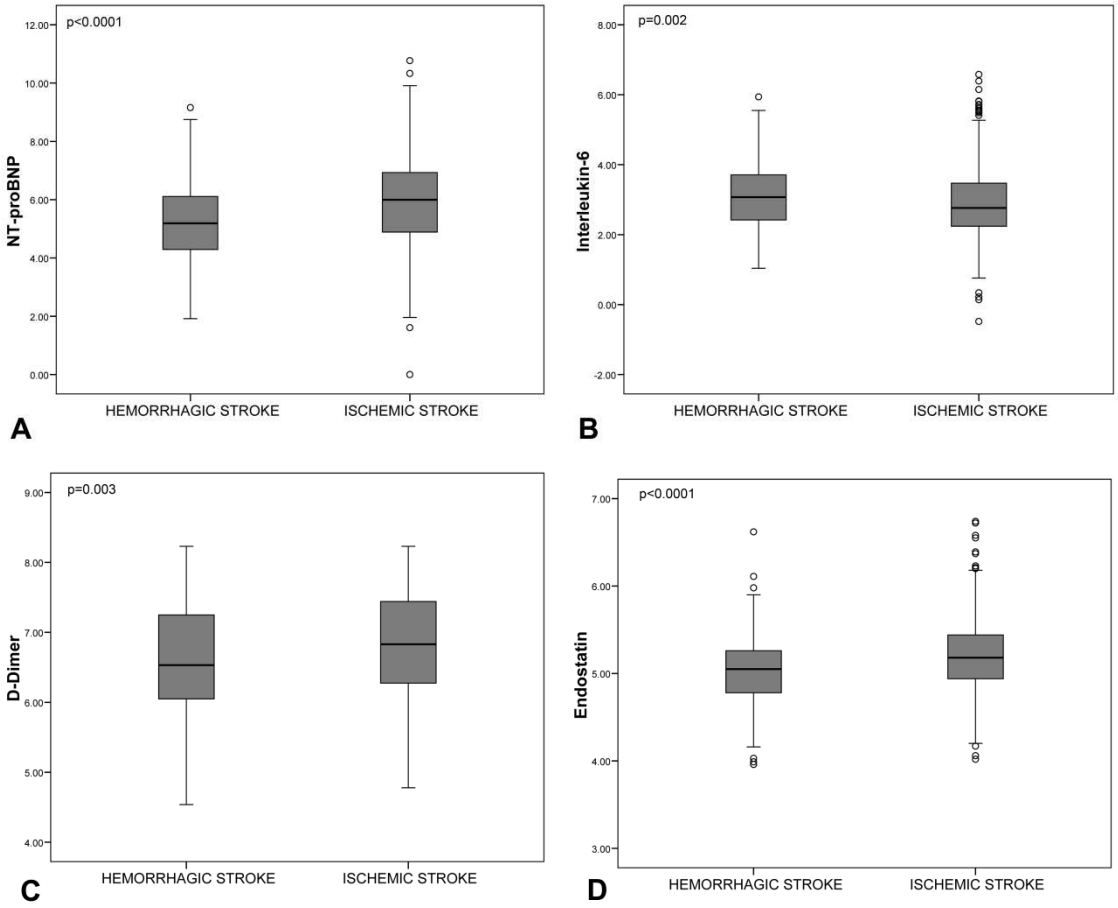
The tables show the final models of the logistic regression analyses for both comparisons, real strokes vs. stroke-mimics (A) and ischemic vs. hemorrhagic stroke (B), after including in first step all variables associated with the dependent variable at a p value >0.1: age, gender, alcohol, arterial hypertension, dyslipidemia, atrial fibrillation, previous stroke, systolic blood pressure (SBP), diastolic blood pressure (DBP), glycemia, National Institutes of Health Stroke Scale (NIHSS, not for model 2), vascular adhesion protein 1 (VAP-1), endostatin, interleukin-6 (IL-6), D-dimer, N-terminal pro B-type natriuretic peptide (NT-proBNP) and growth-related oncogen alpha (GroA) for stroke vs. stroke-mimics and age, gender, alcohol, hypertension, atrial fibrillation (AF), coronary artery disease, systolic blood pressure (SBP), diastolic blood pressure (DBP), glycemia, National Institutes of Health Stroke Scale (NIHSS, not for model 2), N-terminal pro B-type natriuretic peptide (NT-proBNP), endostatin, interleukin-6 (IL-6) and D-dimer for ischemic vs. hemorrhagic stroke.

SUPPLEMENTAL FIGURE I: BIOMARKERS LEVELS IN REAL STROKES VS. STROKE MIMICS



Boxplots show mean ± standard deviation (A) or median (interquartile range) (B-E) of the biomarker's log-transformed levels among patients with final diagnosis of real stroke or stroke-mimicking condition. Just biomarkers differentiating between both conditions are showed. NT-proBNP: N-terminal pro B-type natriuretic peptide; VAP-1: vascular adhesion protein 1.

SUPPLEMENTAL FIGURE II: BIOMARKERS LEVELS IN ISCHEMIC VS. HEMORRHAGIC STROKE



Boxplots show mean ± standard deviation (A) or median (interquartile range) (B-D) of the biomarker's log-transformed levels among patients with final diagnosis of ischemic or hemorrhagic stroke. Just biomarkers differentiating between both conditions are showed. NT-proBNP: N-terminal pro B-type natriuretic peptide.

SUPPLEMETAL TABLE III: BIOMARKERS CUT-OFFS, SENSITIVITY AND SPECIFICITY

A. INTERIM ANALYSIS

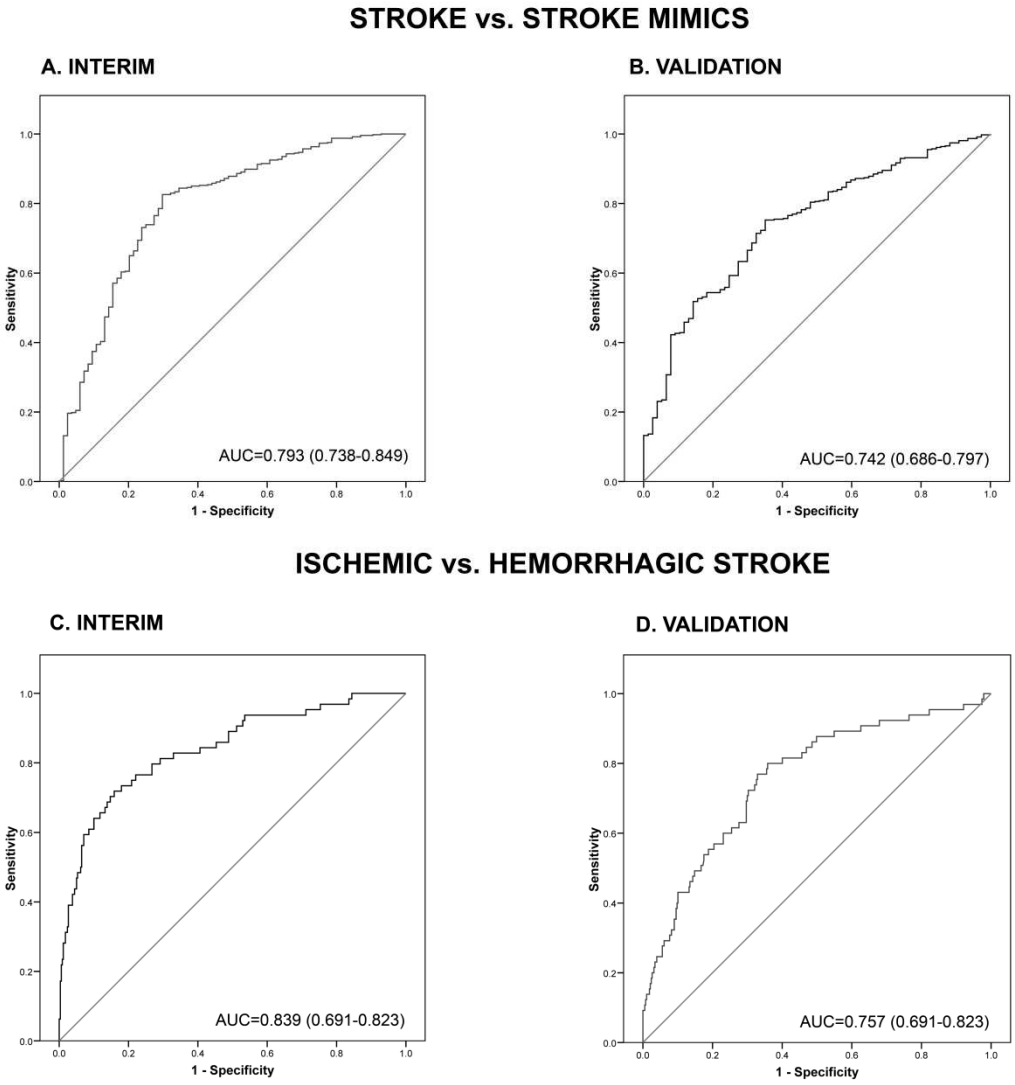
	Cut- Off	Sensitivity	Specificity	Patient Type (% above cut-off)		p
STROKE vs. MIMICS				**Stroke**	**Mimic**	
ApoC-III	5.005	71.9%	42.9.%	49.5%	45.1%	0.002
NT-proBNP	3.465	94.7%	19.2%	95.0%	83.3%	<0.0001
FasL	0.112	88.6%	20.6%	16.1%	23.7%	0.011
ISCHEMIC vs. HEMORRHAGIC STROKE				**Ischemic**	**Hemorrhagic**	
ApoC-III	5.185	65.8%	47.8%	40.4%	35.6%	0.011
GroA	3.245	73.7%	39.6%	65.8%	73.5%	0.014
NT-proBNP	5.695	57.1%	67.0%	57.3%	33.1%	<0.0001
Hsc70	0.114	73.2%	44.0%	86.2%	77.0%	0.003

B. WHOLE COHORT ANALYSIS

	Cut- Off	Sensitivity	Specificity	Patient Type (% above cut-off)		p
STROKE vs. MIMICS				**Stroke**	**Mimic**	
NT-proBNP	4.685	76.9%	43.5%	76.9%	56.3%	<0.0001
IL-6	2.225	76.8%	40.7%	76.7%	59.6%	<0.0001
D-dimer	6.015	82.5%	39.7%	82.5%	60.1%	<0.0001
VAP-1	12.515	74.1%	36.8%	74.1%	63.0%	0.002
Endostatin	4.885	77.2%	39.2%	77.2%	60.6%	<0.0001
ISCHEMIC vs. HEMORRHAGIC STROKE				**Ischemic**	**Hemorrhagic**	
NT-proBNP	4.900	44.8%	74.9%	74.9%	55.2%	<0.0001
IL-6	3.665	28%	80.9%	19.1%	28.3%	0.006
D-dimer	6.095	29.7%	81.3%	81.3%	69.9%	0.001
Endostatin	4.675	18.8%	90.8%	90.7%	81.6%	<0.0001

NT-proBNP: N-terminal pro B-type natriuretic peptide; FasL: Fas ligand; GroA: groth-related oncogen alpha; Hsc70: heat shock 70 kDa protein 8; IL-6: interleukin-6; VAP-1: vascular adhesion protein-1

SUPPLEMENTAL FIGURE III: ROC CURVES IN THE INTERIM AND VALIDATION COHORTS



The figure shows the receiver operating characteristics (ROC) curves corresponding to the logistic regression models described in the manuscript, for the comparison between real strokes and stroke mimics in the interim (A) and validation (B) cohorts and between ischemic and hemorrhagic strokes in the interim (C) and validation (D) cohorts.

SUPPLEMENTAL REFERENCES

1. Montaner J, Mendioroz M, Ribó M, Delgado P, Quintana M, Penalba A, et al. A panel of biomarkers including caspase-3 and D-dimer may differentiate acute stroke from stroke-mimicking conditions in the emergency department. J Intern Med 2011; 270: 166-174
2. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez J-C, et al. PanelomiX: A threshold-based algorithm to create panels of biomarkers. Translational Proteomics 2013; 1: 57-64.