ORIGINAL RESEARCH

Heads and Tails of Natriuretic Peptides: Neuroprotective Role of Brain Natriuretic Peptide

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***Background*—**Besides the relevant role of brain-type natriuretic peptide (BNP) as biomarker of cardioembolic strokes, new experimental evidences suggest that this peptide may mediate neuroprotective effects. In this study, we have evaluated for the first time the clinical association between BNP (by means of proBNP) and good outcome in ischemic stroke patients, and analyzed the effect of blood BNP increase in an ischemic animal model.

***Methods and Results*—**A retrospective study with 2 different cohorts (262 patients in cohort I and 610 in cohort II) from the same prospective stroke registry was performed. proBNP concentration was analyzed within the first 12 hours from stroke onset. The primary predictor variable was functional outcome evaluated by modified Rankin Scale at 3 months. For the experimental study, BNP pretreatment was tested in an ischemic animal model subjected to a transient occlusion of the cerebral artery, and the infarct volume and sensorimotor deficit were evaluated for 14 days. Cardioembolic strokes presented a positive correlation between proBNP concentration and modified Rankin Scale at 3 months; however, noncardioembolic strokes presented a negative correlation. In the logistic regression analysis, noncardioembolic strokes with concentrations of proBNP ≥340 pg/mL were associated with a good outcome. In line with these clinical findings, the experimental study revealed that those BNP pretreated animals presented a reduction on infarct volumes at 24 hours and functional recovery at days 7 and 14 compared with the control groups.

***Conclusions*—**These clinical and experimental evidences support the potential role of BNP as a protective factor against cerebral ischemia. **(*J Am Heart Assoc*. 2017;6:e007329. DOI: 10.1161/JAHA.117.007329.)**

Key Words: brain natriuretic peptide • cardioembolic stroke • neuroprotection • noncardioembolic stroke

It is well known that natriuretic peptides (NPs) play an important role in electrolyte homeostasis and in the control of body water. In opposition to the renin–angiotensin–aldosterone system, NPs produce an excretion of sodium and water, reducing the blood pressure.[1]

Currently, 3 different types of NPs have been described: atrial natriuretic peptide, brain-type natriuretic peptide (BNP), and C-type natriuretic peptide. Atrial natriuretic peptide is released by atrial myocytes of the heart in response to atrial distension. BNP (initially identified in brain) is synthesized largely by the heart ventricles. BNP is first synthesized as preproBNP, which is then converted to proBNP and cleaved for the corin enzyme to produce the active form BNP and the N-terminal piece of proBNP. Like atrial natriuretic peptide, BNP is released in heart stress situations. C-type natriuretic peptide, contrary to atrial natriuretic peptide and BNP, does not have direct natriuretic activity.[1,2]

Clinical studies have reported that proBNP acts as a predictive biomarker of heart failure (BNP has a half-life of ≈20 minutes and is quickly cleared, while proBNP has a half-life of ≈1 to 2 hours, leading to higher circulation levels and slower fluctuations).[3,4] In addition, proBNP has been described as a biomarker in some types of cerebrovascular diseases. Indeed, high levels of proBNP are associated with

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Accompanying Tables S1, S2 and Figure S1 are available at http://jaha.ahajournals.org/content/6/12/e007329/DC1/embed/inline-supplementary-material-1.pdf

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Received August 4, 2017; accepted October 24, 2017.

Clinical Perspective

What Is New?

- Noncardioembolic strokes present a negative correlation between pro-brain-type natriuretic peptide concentration and modified Rankin score at 3 months.
- Patients with noncardioembolic strokes and serum concentrations of pro-brain-type natriuretic peptide ≥340 pg/mL were associated with good outcome.
- Pretreatment with brain-type natriuretic peptide induces a reduction on infarct volumes and functional recovery in a rat model of middle cerebral artery occlusion.

What Are the Clinical Implications?

- Brain-type natriuretic peptide potentially acts as a protective agent against cerebral ischemic injury.

cardioembolic strokes mainly because of atrial fibrillation (AF),[5,6] and can be used to predict the development of atrial fibrillation after first cryptogenic stroke[7] and can act as an independent predictor of mortality after stroke.[8–10]

Despite the relevant role of BNP as a biomarker in heart failure and diagnosis of stroke subtypes, new experimental findings suggest that this peptide also has other beneficial effects on the cardiovascular system through its anti-inflammatory properties, which leads to a reduction of myocardial hypertrophy and atherosclerosis progression.[11–14] In brain, expression of NP receptors have been described in regions such as the cerebral cortex, limbic area, preoptic–hypothalamic regions, cerebellum, or brainstem,[1,15–17] and there are experimental evidences that show NPs may exert neuroprotective effects in cultured cells and in vivo studies.[18–21]

Therefore, based on these experimental findings, our aim was to evaluate, for the first time, the clinical association between BNP (by means of proBNP analysis) and outcome variables in ischemic stroke patients and analyze the effect of blood BNP increase in an ischemic animal model.

Materials and Methods

The data that support the findings of this study are available from the corresponding author upon request.

Study Population and Patient Characteristics

We retrospectively analyzed 2 different cohorts of ischemic stroke patients from the same prospective stroke registry of the Neurovascular Area of the Neurology Department of the University Clinical Hospital of Santiago de Compostela (BICHUS). The registry was approved for the Ethics Committee of Galicia (CEIC). Signed informed consent was obtained from patients before study inclusion. In case the patient was not able to sign, the informed consent was obtained from a relative.

The inclusion criteria used in this study were previously described in detail in a previous study conducted for our group to study the role of BNP as biomarker for stroke.[6] The acute management (diagnostic and treatments) of patients with stroke was performed according to the protocols described by the European Stroke Organization.[22] Stroke subtype was classified as atherothrombotic, cardioembolic, lacunar, or undetermined according to TOAST (Trial of Org 10172 in Acute Stroke Treatment) criteria.[23] For this study we have grouped the patients into 2 groups: cardioembolic and noncardioembolic strokes. The group of noncardioembolic strokes included atherothrombotic strokes and lacunar strokes located in deep territories, while undetermined strokes were excluded for this analysis.

Cohort I included a total of 262 patients, already described in a previous study from our group.[7] Briefly, from January to June 2006, 372 patients were included within the first 12 hours from stroke onset. Patients in a coma or with severe stroke (National Institutes of Health Stroke Scale score [NIHSS] >20) (n=30), with previous disability (defined as modified Rankin Scale score [mRS] ≥2) (n=12), severe systemic disease (n=26), dementia or psychiatric disease (n=8), unstable cardiovascular disease (n=28), or life expectancy <3 months (n=6) were excluded.

In cohort II, a total of 741 patients were initially evaluated from January 2013 to March 2014. Then, 29 patients with NIHSS >20, 16 patients with mRS ≥2, 31 patients with severe systemic disease, 12 patients with dementia or psychiatric disease, 32 with unstable cardiovascular disease, and 8 with life expectancy <3 months were excluded; therefore, a total of 610 patients were finally included. Once the undetermined strokes were excluded, 100 cardioembolic and 78 noncardioembolic strokes were analyzed in cohort I, and 226 cardioembolic and 210 noncardioembolic strokes in cohort II.

Inclusion criteria and variables analyzed were similar in both cohorts; however, patients included in the first cohort were only treated with intravenous thrombolytic therapy, while in the second cohort, patients were treated with intravenous thrombolysis and other recanalization therapies (intraarterial thrombolysis or mechanical thrombectomy). In addition, the reperfusion therapeutic windows for thrombolysis treatment were different for cohort I and cohort II: 3 or 4.5 hours, respectively. Therefore, both cohorts were analyzed separately.

Outcome Variables

Our primary predictor variable was considered the functional outcome evaluated by mRS at 3 months after stroke. We considered good functional outcome a mRS score of <3.

As secondary end point, we evaluated the change (%) of NIHSS at 48 hours with respect to basal situation (at admission) by using the following formula: (NIHSS 48 hours−NIHSS at admission)×100/NIHSS at admission. Likewise, we determined the difference (%) between NIHSS at 3 months and 48 hours after stroke following this equation: (NIHSS 3 at months−NIHSS at 48 hours)×100/NIHSS at 48 hours.

Early neurological deterioration was evaluated as secondary outcome and defined as a NIHSS increment of ≥4 points within the first 48 hours from stroke onset.

On the other hand, although the aim of this study was to analyze the association between the proBNP levels and outcome variables in ischemic stroke patients, because of the role of this peptide as biomarker of atrial fibrillation,[5] we also analyzed the independent association between this biomarker with cardioembolic stroke pathogenesis.

Laboratory Tests

Blood samples on admission were collected in glass chemistry tubes, centrifuged at 3000*g* for 10 minutes, and immediately frozen and stored at −80°C. Serum proBNP levels were measured by electrochemiluminescence immunoassay (ELECSYS 2010 System; Roche Diagnostics GmbH, Mannheim, Germany). Determinations were performed in an independent laboratory blinded to clinical data. The intra-assay and interassay coefficients of variation were <5%.

Animals

All experimental protocols were approved by the local Animal Care Committee according to the European Union (EU) rules (86/609/CEE, 2003/65/CE, and 2010/63/EU). Male Sprague-Dawley rats weighing between 280 and 330 g were used. Animals were housed individually, in stable environmental conditions (environmental temperature of 23°C, relative humidity of 40%, and a light–dark cycle of 12 hours), and free access to food and water. Eight animals per group were required to detect this difference with a power (1−b) of 0.8 and α=0.05. N was calculated using EPIDAT software (http://www.sergas.es/Saude-publica/EPIDAT-4-2) and based on previous studies.[21]

BNP Pharmacokinetic Study in Animals

Initially, a pharmacokinetic study with BNP was performed in an independent group of healthy animals. In the control group (n=3), animals were treated with the vehicle solution (intravenously). In the treated group (n=3), animals were treated (intravenously) with 12 nmol/kg of human BNP (hBNP) (Genscript, NJ), at a dose that has been previously demonstrated to increase the endogenous levels of BNP in rats.[24] For BNP dissolution, powder was dissolved in a minimal amount of 50 mmol/L Na-acetate buffer, and then resuspended in saline, and the pH was adjusted to 7.4. hBNP concentration was adjusted to inject 1 mL of treatment per animal. Blood samples (from vein tail, 200–300 μL) were obtained in basal conditions (before treatment administration) and 5, 15, 30, 45 minutes and 1 and 2 hours after treatment injection. Blood BNP levels were determined by means of BNP EIA Kit (RAB0386, Sigma).

Cerebral Ischemic Animal Model

Cerebral ischemia was induced by intraluminal occlusion of the middle cerebral artery (MCA), following the method described by Longa et al.[25] All surgical procedures were performed with animals under sevoflurane anesthesia (6% induction and 4% maintenance in a gas mixture of 70% NO_2 and 30% O_2). During surgery, all animals were subjected to temperature control, maintaining rectal temperature at 37±0.5°C by a thermostat-controlled electric pad (NeosBiotec, Spain).

The commercial sutures used for MCA occlusion had a silicon-rubber-coated head of 350-μm diameter and 1.5-mm length (Doccol, MA). Cerebral blood flow was monitored with a Periflux 5000 laser-Doppler system (PerimedAB, Sweden) by placing a Doppler probe (model 411; PerimedAB, Sweden) in the parietal bone surface near the sagittal crest, under the temporal muscle. MCA occlusion was determined by Doppler signal reduction, and after that, animals were carefully moved from the surgical bench to a magnetic resonance (MR) scanner, in order to determine the basal ischemic lesion by means of apparent diffusion coefficient (ADC) maps. Angiography imaging was also performed to confirm that the artery remained occluded over the MR study. After basal MR analysis, animals were returned to the surgical bench and the Doppler probe was repositioned. Reperfusion was performed 45 minutes after the arterial occlusion.

The following animal exclusion was used.

1. Cerebral blood flow reduction <70% when performing the occlusion, measured by Doppler.
2. Basal lesion volume <25% or >45% of the ipsilateral hemisphere measured with ADC maps.
3. Absence of reperfusion or long reperfusion time (>10 minutes to recover 50% of the basal cerebral blood flow) after artery occlusion, measured by Doppler, once the animal is returned to the surgical bench.

All animals that were excluded or that died were replaced until the 2 groups reached n=8/each.

A total of 26 animals were used in the BNP protective study, but 8 were discarded because they did not pass the inclusion criteria and 2 died during the surgery.

Experimental Groups in Ischemic Animals

Two experimental groups were performed.

1. Control group (n=8). In this group, before MCA occlusion, animals were treated (intravenously) with 1 mL of drug vehicle.
2. hBNP-treated group (n=8). Animals were treated intravenously) before MCA occlusion with the same dose of 12 nmol/kg hBNP used previously in the pharmacokinetic study.

In both groups, brain injury was determined during MCA occlusion, and at 24 hours, 7 days, and 14 days after ischemia by means of MR imaging. Functional outcome was examined by the rotarod and grip tests before ischemic surgery, and 7 and 14 days after surgery.

Animals were randomly assigned to treatment groups of the study; researchers were blinded to treatment administration and to treatments during outcome assessment; and the body temperature of animals was controlled during the ischemic period.

Functional Tests

Functional tests were performed using a rotarod (Ugo Basile SRL, Varese, Italy) and a grip (Bioseb, Vitrolles, France) apparatus.

For the rotarod test, rats were previously trained for 3 days. Rotarod protocol consisted of placing the animals over the static cylinder, allowing the animals to become familiarized with the apparatus, and after a moment, set the cylinder to run progressively from 5 rpm until 20 rpm. After 3 days of training, animals used to stay in the rotarod between 100 and 120 s before placing them again inside their cages. Once trained, when test had to be done, each animal was placed 3 times (with 3–5 minutes of rest between trials) into the rotarod at 20 rpm, and the time they remained on it was measured.

Grip test consisted of a small leaky rack attached to an apparatus that measured the grip strength. To perform the test, animals were handled by the tail and carefully approached to the grip rack, allowing them to grab on it just with the front legs. Then the animal was pulled back, so the strength with which they grabbed was registered by the grip apparatus.

Magnetic Resonance Imaging Assessment and Magnetic Resonance Imaging Data Analysis

All studies were conducted on a 9.4-T horizontal bore magnet (Bruker BioSpin, Ettlingen, Germany) with 440-mT/m gradients and a combination of a linear birdcage resonator (7 cm in diameter) for signal transmission and a 2×2 surface coil array for signal detection.

Basal ischemic lesion during MCA occlusion was determined by counting pixels with ADC values below a threshold in the ipsilateral brain hemisphere. Values of ADC in the healthy rat brain normally do not fall below 0.55×10^{-3} mm^2/s; this threshold provides a convenient means of segmenting abnormal tissue.

ADC maps were obtained from diffusion-weighted images using a spin echo echo-planar imaging sequence with the following acquisition parameters: echo time (ET)=26.91 ms, repetition time=4 s, spectral bandwidth=200 kHz, 7 b-values of 0, 300, 600, 900, 1200, 1600, and 2000 s/mm^2, flip angle=90°, number of averages=4, 14 consecutive slices of 1 mm, 24×16 mm^2 field of view (FOV) (with saturation bands to suppress signal outside this FOV), a matrix size of 96×64 (isotropic in-plane resolution of 250×250 μm/pixel), and implemented with fat suppression option. MCA occlusion status was evaluated in a noninvasive manner with the time-of-flight magnetic resonance angiography. A time-of-flight magnetic resonance angiography scan was performed with a 3-dimensional-Flash sequence with an echo time=2.5 ms, repetition time=15 ms, flip angle=20°, number of averages=2, spectral bandwidth=98 kHz, 1 slice of 14 mm, $30.72\times30.72\times14$ mm^3 FOV (with saturation bands to suppress signal outside this FOV), a matrix size of $256\times256\times58$ (resolution of $120\times120\times241$ μm/pixel) and implemented without fat suppression option. The progression of ischemic lesions and infarct volumes was determined from T2 maps calculated from T2-weighted images acquired 1, 7, and 14 days after the onset of ischemia using a multislice multiecho sequence: with an echo time=9 ms, repetition time=3 s, 16 echoes with 9-ms echo spacing, flip angle=180°, number of averages=2, spectral bandwidth=75 kHz, 14 slices of 1 mm, 19.2×19.2 mm^2 FOV (with saturation bands to suppress signal outside this FOV), a matrix size of 192×192 (isotropic in-plane resolution of 100×100 μm/pixel), and implemented without fat suppression option.

Images were processed using ImageJ (Rasband WS, National Institutes of Health, Bethesda, MD) on an independent computer workstation. Infarct volumes of ischemic animals were determined from quantitative ADC maps and T2 relaxation maps. Lesion volume as a percentage of the ipsilateral hemispheric volume was calculated as (lesion volume [mm^3]/ipsilateral hemispheric volume [mm^3])×100.

Statistical Analyses

Results were expressed as percentages for categorical variables and as mean (SD) or median (quartiles) for the continuous variables, depending on whether their distribution was normal or not. The Kolmogorov–Smirnov test was used for testing the normality of the distribution.

Comparison between the different groups was calculated by means of χ^2 test for categorical variables and Student *t* test, Mann–Whitney test, or ANOVA test in case of continuous variables. Correlation between variables was performed by means of Spearman's coefficient. Receiver operating characteristic curves were used to establish the cutoff points of proBNP levels that optimally predicted the cardioembolic origin of stroke. Odds ratios were adjusted by significant variables of the bivariate analysis. Results were expressed as odds ratios with the corresponding 95% confidence intervals (95% CI). A $P<0.05$ was considered to be statistically significant in all tests.

The statistical analysis was separately performed in each cohort, and subsequently in both cohorts together.

For the experimental study, all data are presented as the mean and standard error of the mean (mean±SEM). Data were first examined to assess distribution using the D'Agostino and Pearson omnibus normality test. One-way or 2-way ANOVA followed by post hoc Bonferroni evaluation were used both for infarct volumes and functional test results to determine significant differences. Statistical significance was set at $P<0.05$.

The statistical analysis was conducted in SPSS 20.0 (IBM, Chicago, IL) for Mac.

Results

Clinical Study

Descriptive analysis of the clinical, biochemical, and outcome variables of 2 studied cohorts of stroke patients (Table S1) shows that in cohort II, patients were older ($P<0.0001$) and a higher number of patients were subjected to thrombolytic treatment ($P=0.010$). Basal proBNP levels, determined within the first 12 hours from stroke onset, were similar in both cohorts (885.0±700.6 pg/mL versus 884.4±744.5 pg/mL, $P=0.993$).

proBNP concentration was higher in the cardioembolic strokes compared with noncardioembolic strokes in the 2 cohorts analyzed. In addition, cardioembolic strokes with higher proBNP concentration had a worse outcome at 3 months, while in the noncardioembolic strokes, higher proBNP concentration was associated with a better outcome at 3 months, as indicated in Table. Indeed, when we analyzed the correlation between proBNP concentration and mRS at 3 months, cardioembolic strokes presented a positive correlation between proBNP concentration and mRS at 3 months; however, noncardioembolic strokes presented a negative correlation in cohorts I and II and in the analysis of cohorts I and II (Figure 1).

Receiver operating characteristic curves were used to establish the cutoff point of proBNP concentration that predicts the cardioembolic strokes. In cohort I, concentration ≥340 pg/mL of proBNP predicted a cardioembolic stroke with a specificity of 87% and a sensitivity of 83% (area under the curve 0.92; 95% CI, 0.83–0.96; $P<0.0001$), and in cohort II concentration ≥340 pg/mL of proBNP predicted a cardioembolic stroke with a specificity of 84% and a sensitivity of 79% (area under the curve 0.89; 95% CI, 0.79–0.95; $P<0.0001$). Finally, when both cohorts were analyzed together, proBNP levels ≥340 pg/mL predict a cardioembolic stroke with a specificity of 85% and a sensitivity of 81% (area under the curve 0.89; 95% CI, 0.81–0.92; $P<0.0001$) (Figure 2).

75.4% of noncardioembolic strokes with a proBNP levels ≥340 pg/mL had good functional outcome at 3 months.

Table. proBNP (pg/mL) Levels According to Functional Outcome at 3 Months in Stroke Patients

Patients	Good Outcome	Poor Outcome	*P* Value
Cohort I (proBNP, pg/mL)			
All patients	920.6±617.7	804.2±799.5	0.222
Cardioembolic strokes	1104.8±565.5	1588.6±490.1	<0.0001
Noncardioembolic strokes	832.5±625.1	208.0±337.3	<0.0001
Cohort II (proBNP, pg/mL)			
All patients	783.0±616.8	988.4±844.1	0.001
Cardioembolic strokes	761.1±646.6	1622.2±670.6	<0.0001
Noncardioembolic strokes	791.0±606.8	421.9±517.3	<0.0001
Cohort I and cohort II (proBNP, pg/mL)			
All patients	824.9±619.6	946.9±836.8	0.016
Cardioembolic strokes	880.2±638.9	1615.2±635.8	<0.0001
Noncardioembolic strokes	802.9±611.4	371.0±488.4	<0.0001

proBNP indicates pro-brain natriuretic peptide.

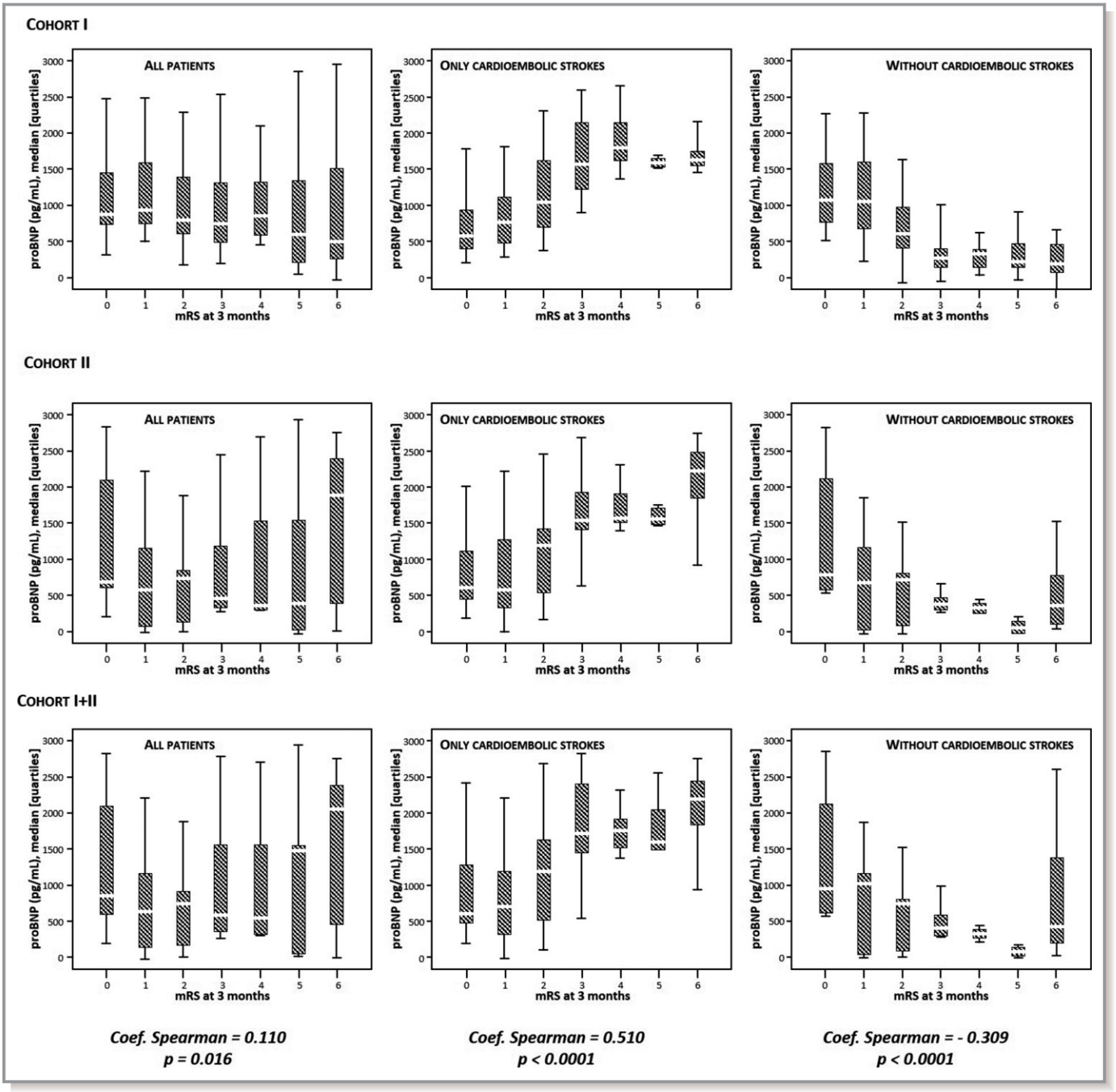

Figure 1. Correlations between pro-brain natriuretic peptide (proBNP) concentration and modified Rankin Scale (mRS) score at 3 months after stroke in cohort I, cohort II, and cohorts I+II in all patients, in cardioembolic stroke patients, and noncardioembolic stroke patients. Spearman's coefficient and *P* values were calculated for cohorts I+II.

However, only 38.4% of patients who showed proBNP levels <340 pg/mL had good functional outcome at 3 months (Figure 3). In the logistic regression analysis, proBNP levels ≥340 pg/mL were associated with good functional outcome (odds ratio 3.45; 95% CI, 2.12–5.62; *P*<0.0001) in noncardioembolic strokes after adjusting by age, NIHSS at admission, body temperature at admission, systolic and diastolic blood pressure at admission, glycemia at admission, and thrombolysis (Table S2).

No differences were found regarding early neurological deterioration between cardioembolic and noncardioembolic strokes (4.6% versus 3.5%, *P*=0.460). However, early neurological deterioration was higher in those cardioembolic stroke patients with proBNP levels <340 pg/mL compared with those patients with levels ≥340 pg/mL (5.2% versus 2.6%, *P*=0.460). (*P*=0.043). Similar results were observed in noncardioembolic strokes (5.6% versus 2.1%, *P*=0.036).

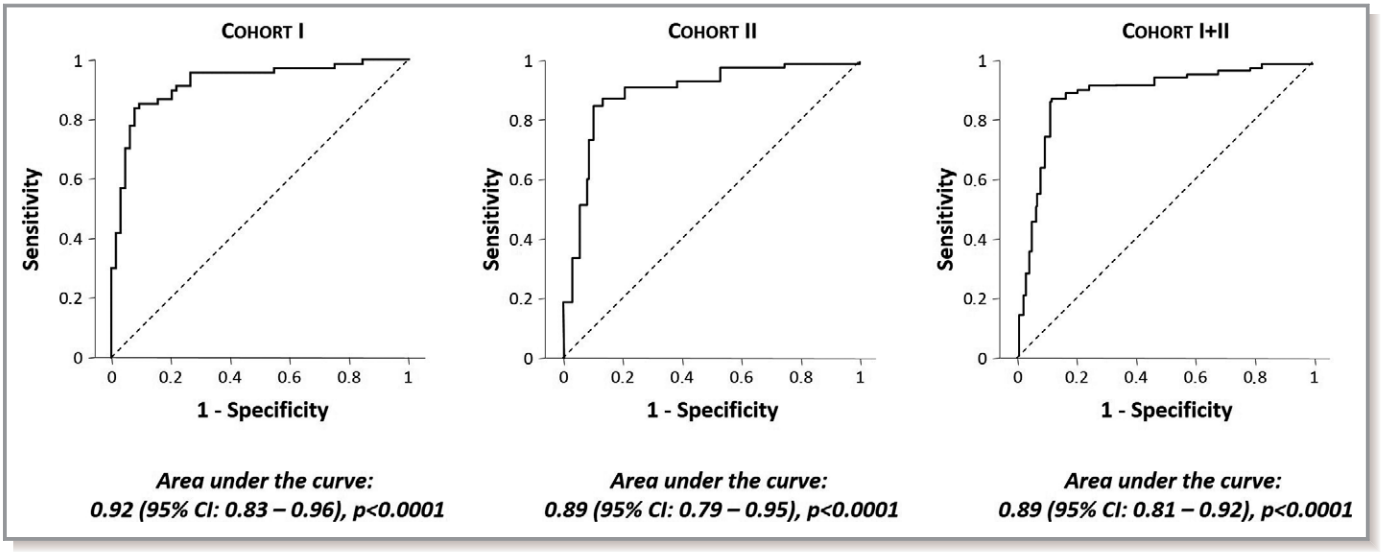

Figure 2. Receiver operating characteristic curves analysis of the cutoff point of pro-brain natriuretic peptide concentration to predict cardioembolic strokes in cohorts I, II, and I+II. CI indicates confidence interval.

Although evolution of the neurological deficits within the first 48 hours was similar in both cardioembolic and noncardioembolic strokes, a higher neurological deterioration within the first 48 hours was observed in those noncardioembolic stroke patients with lower proBNP levels (−45.8±151.8% versus −108.9±205.5%; P=0.001; proBNP <340 and ≥340 pg/mL, respectively) (Figure S1A). In case of cardioembolic strokes, there were no differences between patients with proBNP <340 and ≥340 pg/mL (P=0.765). Likewise, neurological deficits analyzed between 48 hours and 3 months after stroke were similar in all groups (Figure S1B).

Experimental Study

In the BNP pharmacokinetic study, previously we tested that the dose used (12 nmol/kg) induced an acute significant increase of blood BNP levels (Figure 4). When animals were pretreated with hBNP immediately before ischemia induction, this drug caused a reduction of infarct volume at 1, 7, and 14 days after ischemia compared with the control group, although the significant difference was achieved at 1 day after ischemia (95% CI of diff=−13.21 to −0.49; P<0.05) (Figure 5). All animals included in the BNP protective study presented similar ischemic volume (36.42±2.37% versus 36.30±2.03%; control and treated group, respectively) at the moment of the MCA occlusion, which suggests that all animals were submitted to the same ischemic damage. Besides the differences of infarct volume, significant differences were also found in the functional analysis at 7 (95% CI of diff=15.39–72.21; P<0.01) and 14 days (95% CI of diff=12.98–69.80; P<0.01) compared with the control group using the rotarod test,

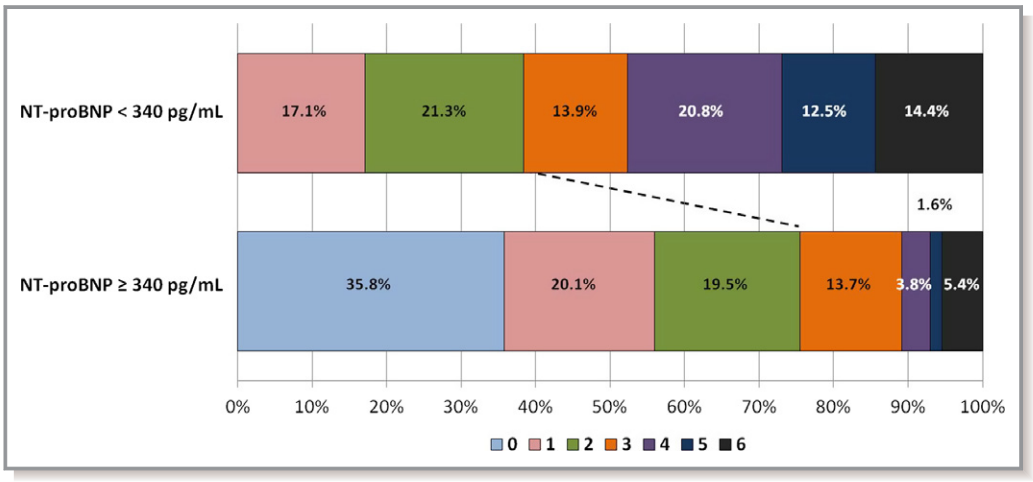

Figure 3. Shift distribution of the modified Rankin Scale scores at 3 months in noncardioembolic stroke patients (in both cohorts). NT-proBNP indicates N-terminal pro-brain natriuretic peptide.

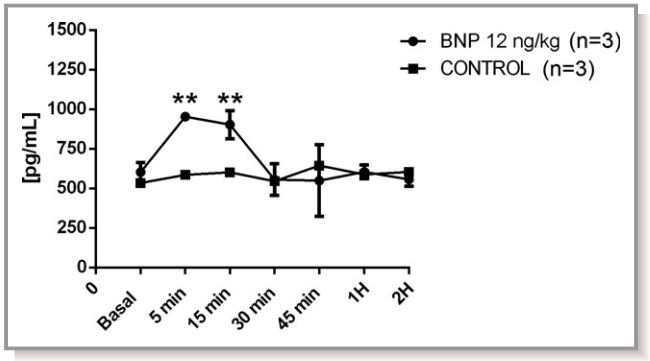

Figure 4. Brain natriuretic peptide (BNP) pharmacokinetic study. In the control group, animals were treated with the vehicle solution (intravenous). In the treated group, animals were treated (intravenous) with 12 nmol/kg of human BNP. Data are shown as mean±SEM. $^{**}P<0.01$ with respect to the control, (n=3/group).

although no differences were observed in the grip test (Figure 6A and 6B, respectively).

Discussion

NPs play an important physiological function in the body as endocrine and paracrine molecules involved in the control of water and therefore in the regulation of arterial pressure.[1,26] Experimental evidences have also described the protective effect of NPs in cerebral injury[18–21]; however, although the study of NPs (mainly BNP) has been focused as a biomarker of heart failure[3,26] and biomarker of cardioembolic stroke,[5–10] a clinical analysis about the protective effect of BNP in stroke that confirms the beneficial effect of these molecules has not been performed so far.

The clinical results shown here support the potential endogenous protective role of BNP in ischemic stroke pathology. Thus, we have observed that concentration of proBNP ≥340 pg/mL was independently associated with good outcome at 3 months in noncardioembolic stroke patients.

To validate the potential protective effect observed in those noncardioembolic strokes with higher proBNP levels in the clinical study, hBNP (active form) was administered in ischemic animal models. Our experimental study confirmed the clinical findings as animals with higher blood levels of hBNP before ischemia presented significant infarct volume reduction and a better sensorimotor recovery. These results were also in line with a previous experimental study[19] in an animal model of intracerebral hemorrhage and traumatic brain injury that describes that the intravenous administration of nesiritide (a human recombinant form of BNP) improved the cerebral blood flow and reduced the inflammation and brain injury reflected in a better functional outcome, supporting the hypothesis that NPs can act as an endogenous protective mechanism in the brain against injury.[1]

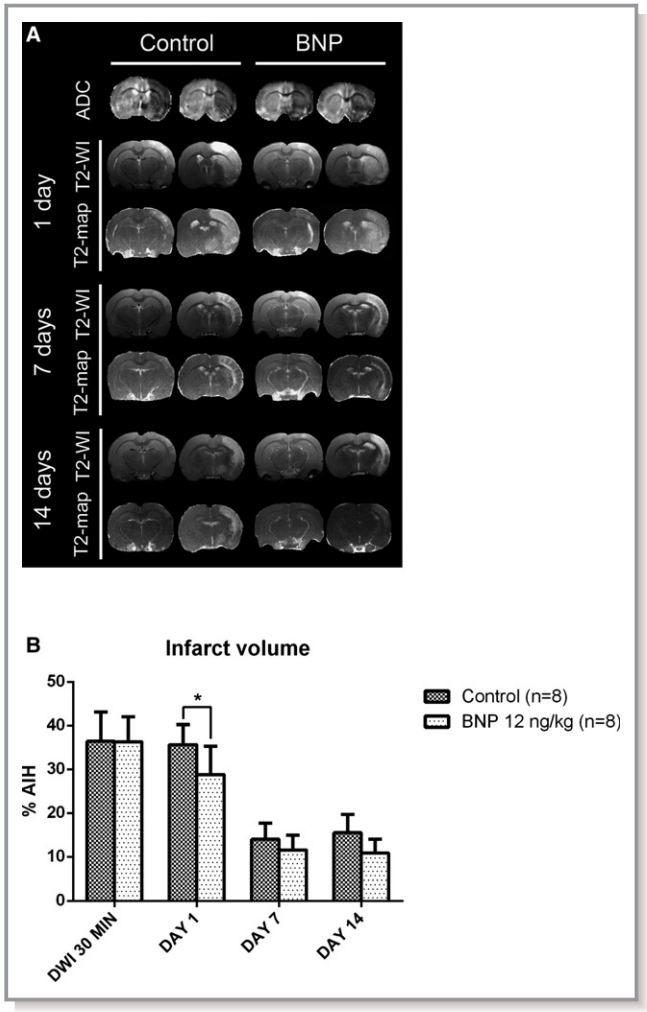

Figure 5. Magnetic resonance imaging (MRI) assessments of ischemic injury evolution. Apparent diffusion coefficient (ADC) maps were recorded during cerebral artery occlusion to ensure that all animals included in the study were subjected to similar levels of ischemic damage. Lesion volume evolution was assessed using T2-weighted images recorded 24 hours, 7 days, and 14 days after ischemia induction. A, MRI assessment of control (vehicle) and treated animals with BNP (12 nmol/kg) before cerebral artery occlusion. B, Quantitative analysis of lesion volumes adjusted to the ipsilateral hemisphere (%). Data are shown as mean±SEM. $^{*}P<0.05$ with respect to the control, (n=8/group). AIH indicates area of injured hemisphere; BNP, brain natriuretic peptide; DWI, diffusion-weighted imaging.

Based on the protective role of BNP defended here, it is necessary to discuss why cardioembolic strokes with higher proBNP levels showed worse outcome than noncardioembolic strokes.[5–7] Cardioembolic strokes are more severe and have worse outcome than noncardioembolic strokes[27,28]; therefore, it is tentatively postulated that the beneficial effect of BNP could be masked in these subtypes of strokes.

On the other hand, our data suggest that the protective effect of proBNP in noncardioembolic strokes is early and not longer than 48 hours after stroke onset, mainly in

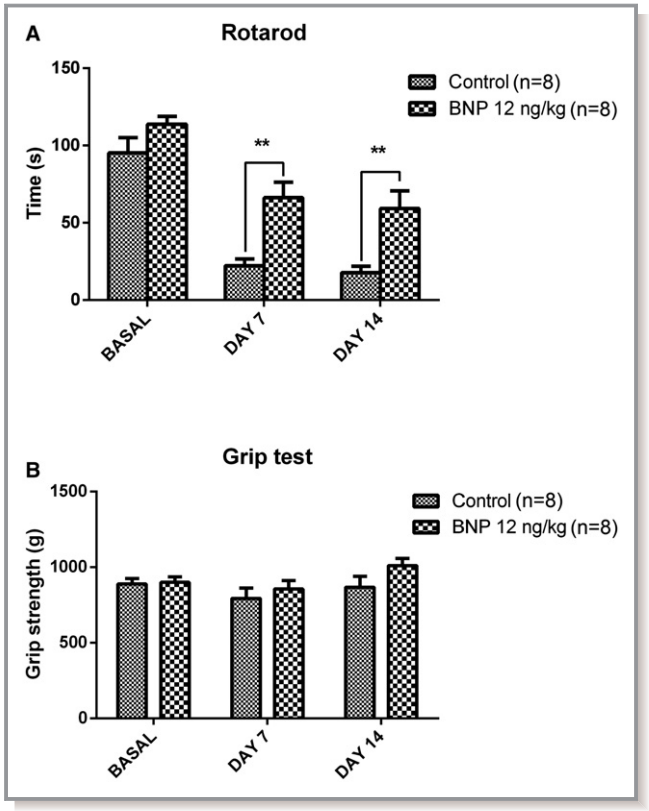

Figure 6. Assessment of sensorimotor function using rotarod test (A) and grip test (B). Functional tests were performed before ischemic injury (baseline) 7 and 14 days after injury in control (vehicle) and treated animals with brain natriuretic peptide (BNP) (12 nmol/kg). Data are shown as mean±SEM. $^{**}P<0.01$ with respect to the control (n=8/group).

noncardioembolic strokes, as the decrease of NIHSS score is not observed between 48 hours and 3 months after stroke. These clinical results are also in consonance with the experimental study, as the higher protective effect evaluated on infarct volume was found mainly at 24 hours after ischemia induction.

BNP receptors are widely expressed in the brain, and BNP has been found in different cerebral regions such as hypothalamus or cerebral cortex,[1,15,17,28] for instance. However, BNP-mRNA has not been detected in cerebral tissue, suggesting a peripheral origin of this peptide.[1,29,30] In vitro and in vivo models of cerebral injury, BNP, and other NPs treatments have demonstrated that the neuroprotective effect observed was mediated through the cGMP pathway, which reduces the sodium and water content in brain and reduces the neurotoxicity caused by overexcitation of *N*-methyl-D-aspartate receptors and inflammation.[18,20,21] However, to date it is not clear whether the neuroprotective effect of NPs is mediated by a direct effect on cerebral NPs receptors or because of an indirect effect on the systemic system.

This study has a limitation that should be taken into consideration. The clinical analysis was performed with cohorts of patients that have been analyzed retrospectively; therefore, the analysis could be influenced by unaccounted variables. On the other hand, pretreatment with BNP in our ischemic animal model of stroke showed only a transient reduction (at 24 hours) in infarct volume. However, a significant and permanent beneficial effect on functional improvement was observed using the rotarod test, which reflects the potential beneficial effect of the BNP.

In conclusion, these clinical and experimental evidences suggest the potential role of BNP as a protective endogenous factor against cerebral ischemia.

Sources of Funding

This study has been partially supported by grants from Instituto de Salud Carlos III (PI13/00292, PI14/01879), Spanish Research Network on Cerebrovascular Diseases RETICS-INVICTUS (RD12/0014), The Ministry of Economy and Competitiveness of Spain (SAF2014-56336-R), Xunta de Galicia (Consellería Educación GRC2014/027), and the European Union program FEDER. Furthermore, Campos (CP14/00154) and Sobrino (CP12/03121) are recipients of a research contract from Miguel Servet Program of Instituto de Salud Carlos III. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the article.

Disclosures

None.

References

1. Hodes A, Lichtstein D. Natriuretic hormones in brain function. *Front Endocrinol (Lausanne)*. 2014;5:201.
2. Pandey KN. Guanylyl cyclase/atrial natriuretic peptide receptor-A: role in the pathophysiology of cardiovascular regulation. *Can J Physiol Pharmacol*. 2011;89:557–573.
3. Daniels LB, Maisel AS. Natriuretic peptides. *J Am Coll Cardiol*. 2007;50:2357–2368.
4. Rubattu S, Sciarretta S, Valenti V, Stanzione R, Volpe M. Natriuretic peptides: an update on bioactivity, potential therapeutic use, and implication in cardiovascular diseases. *Am J Hypertens*. 2008;21:733–741.
5. Llombart V, Antolin-Fontes A, Bustamante A, Giralt D, Rost NS, Furie K, Shibazaki K, Biteker M, Castillo J, Rodriguez-Yanez M, Fonseca AC, Watanabe T, Purroy F, Zhixin W, Etgen T, Hosomi N, Jafarian Kerman SR, Sharma JC, Knauer C, Santamarina E, Giannakoulas G, Garcia-Berrocoso T, Montaner J. B-type natriuretic peptides help in cardioembolic stroke diagnosis: pooled data meta-analysis. *Stroke*. 2015;46:1187–1195.
6. Rodriguez-Yanez M, Sobrino T, Blanco M, de la Ossa NP, Brea D, Rodriguez-Gonzalez R, Leira R, Davalos A, Castillo J. High serum levels of pro-brain natriuretic peptide (pro BNP) identify cardioembolic origin in undetermined stroke. *Dis Markers*. 2009;26:189–195.
7. Rodriguez-Yanez M, Arias-Rivas S, Santamaria-Cadavid M, Sobrino T, Castillo J, Blanco M. High pro-BNP levels predict the occurrence of atrial fibrillation after cryptogenic stroke. *Neurology*. 2013;81:444–447.
8. Chaudhuri JR, Sharma VK, Mridula KR, Balaraju B, Bandaru VC. Association of plasma brain natriuretic peptide levels in acute ischemic stroke subtypes and outcome. *J Stroke Cerebrovasc Dis*. 2015;24:485–491.

9. Duello KM, Nagel JP, Blackshear JL, Freeman WD, Montaner J, Garcia-Berrocoso T. B-type natriuretic peptides and mortality after stroke: a systematic review and meta-analysis. *Neurology*. 2014;83:292–293.
10. Greisenegger S, Segal HC, Burgess AI, Poole DL, Mehta Z, Rothwell PM. Biomarkers and mortality after transient ischemic attack and minor ischemic stroke: population-based study. *Stroke*. 2015;46:659–666.
11. Alexander MR, Knowles JW, Nishikimi T, Maeda N. Increased atherosclerosis and smooth muscle cell hypertrophy in natriuretic peptide receptor A-/- apolipoprotein E-/- mice. *Arterioscler Thromb Vasc Biol*. 2003;23:1077–1082.
12. Kook H, Itoh H, Choi BS, Sawada N, Doi K, Hwang TJ, Kim KK, Arai H, Baik YH, Nakao K. Physiological concentration of atrial natriuretic peptide induces endothelial regeneration in vitro. *Am J Physiol Heart Circ Physiol*. 2003;284: H1388–H1397.
13. Molkentin JD. A friend within the heart: natriuretic peptide receptor signaling. *J Clin Invest*. 2003;111:1275–1277.
14. Moro C, Klimcakova E, Lolmede K, Berlan M, Lafontan M, Stich V, Bouloumie A, Galitzky J, Arner P, Langin D. Atrial natriuretic peptide inhibits the production of adipokines and cytokines linked to inflammation and insulin resistance in human subcutaneous adipose tissue. *Diabetologia*. 2007;50:1038–1047.
15. Herman JP, Dolgas CM, Rucker D, Langub MC Jr. Localization of natriuretic peptide-activated guanylate cyclase mRNAs in the rat brain. *J Comp Neurol*. 1996;369:165–187.
16. Marei HE. Fine structural and immunohistochemical localization of cardiac hormones (ANP) in the right atrium and hypothalamus of the white rat. *Eur J Morphol*. 2002;40:37–41.
17. McKenzie JC, Juan YW, Thomas CR, Berman NE, Klein RM. Atrial natriuretic peptide-like immunoreactivity in neurons and astrocytes of human cerebellum and inferior olivary complex. *J Histochem Cytochem*. 2001;49:1453–1467.
18. Fiscus RR, Tu AW, Chew SB. Natriuretic peptides inhibit apoptosis and prolong the survival of serum-deprived PC12 cells. *Neuroreport*. 2001;12:185–189.
19. James ML, Wang H, Venkatraman T, Song P, Lascola CD, Laskowitz DT. Brain natriuretic peptide improves long-term functional recovery after acute CNS injury in mice. *J Neurotrauma*. 2010;27:217–228.
20. Kuribayashi K, Kitaoka Y, Kumai T, Munemasa Y, Kitaoka Y, Isenoumi K, Motoki M, Kogo J, Hayashi Y, Kobayashi S, Ueno S. Neuroprotective effect of atrial natriuretic peptide against NMDA-induced neurotoxicity in the rat retina. *Brain Res*. 2006;1071:34–41.
21. Wiggins AK, Shen PJ, Gundlach AL. Atrial natriuretic peptide expression is increased in rat cerebral cortex following spreading depression: possible contribution to sd-induced neuroprotection. *Neuroscience*. 2003;118:715–726.
22. European Stroke Organisation Executive C, Committee ESOW. Guidelines for management of ischaemic stroke and transient ischaemic attack 2008. *Cerebrovasc Dis*. 2008;25:457–507.
23. Adams HP Jr, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, Marsh EE III. Classification of subtype of acute ischemic stroke. Definitions for use in a multicenter clinical trial. Toast. Trial of Org 10172 in acute stroke treatment. *Stroke*. 1993;24:35–41.
24. Semenov AG, Seferian KR, Tamm NN, Artem'eva MM, Postnikov AB, Bereznikova AV, Kara AN, Medvedeva NA, Katrukha AG. Human pro-B-type natriuretic peptide is processed in the circulation in a rat model. *Clin Chem*. 2011;57:883–890.
25. Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke*. 1989;20:84–91.
26. Cea LB. Natriuretic peptide family: new aspects. *Curr Med Chem Cardiovasc Hematol Agents*. 2005;3:87–98.
27. Lee KJ, Jung KH, Byun JI, Kim JM, Roh JK. Infarct pattern and clinical outcome in acute ischemic stroke following middle cerebral artery occlusion. *Cerebrovasc Dis*. 2014;38:31–38.
28. Nigro N, Wildi K, Mueller C, Schuetz P, Mueller B, Fluri F, Christ-Crain M, Katan M. BNP but not s-cTnIn is associated with cardioembolic aetiology and predicts short and long term prognosis after cerebrovascular events. *PLoS One*. 2014;9:e102704.
29. Abdelalim EM, Takada T, Torii R, Tooyama I. Molecular cloning of BNP from heart and its immunohistochemical localization in the hypothalamus of monkey. *Peptides*. 2006;27:1886–1893.
30. Langub MC Jr, Watson RE Jr, Herman JP. Distribution of natriuretic peptide precursor mRNAs in the rat brain. *J Comp Neurol*. 1995;356:183–199.

SUPPLEMENTAL MATERIAL

Table S1. Descriptive analysis of clinical, biochemical and outcome variables of the two studied cohorts.

VARIABLE	Cohort I n = 262	Cohort II n = 610	*p*
Age (years)	69.8 ± 11.8	73.5 ± 13.3	< 0.0001
Sex (males, %)	58.8	52.5	0.103
Vascular risk factors			
History of hypertension (%)	56.5	62.5	0.097
History of diabetes (%)	19.8	22.7	0.373
History of hyperlipidemia (%)	22.9	28.3	0.096
History of heart disease (%)	11.5	15.0	0.201
History of atrial fibrillation (%)	22.1	20.7	0.652
Prior stroke or transient ischemic attack (TIA) (%)	13.0	13.5	0.914
Smoke habit (%)	21.4	24.7	0.339
Biochemical parameters at admission			
Body temperature (ºC)	36.4 ± 0.4	36.3 ± 0.8	0.067
Systolic Blood Pressure (mm Hg)	148.5 ± 23.1	151.7 ± 27.1	0.091
Diastolic Blood Pressure (mm Hg)	79.7 ± 14.8	80.9 ± 15.5	0.283
Glucose levels (mg/dL)	131.7 ± 46.6	136.9 ± 51.9	0.172
Platelet counts (x10^3/mL)	233.9 ± 75.4	231.5 ± 80.9	0.670
Fibrinogen levels (mg/dL)	404.4 ± 128.5	401.6 ± 98.5	0.760
proBrain Natriuretic Peptide (proBNP) levels (pg/mL)	885.0 ± 700.6	884.6 ± 744.5	0.993

Clinical variables			
National Institutes of Healt Stroke Scale			
(NIHSS) at admission	9 [6, 15]	5 [2, 12]	0.343
NIHSS at 24 hours	6 [2, 11]	4 [1, 9]	0.832
NIHSS at 48 hours	5 [2, 10]	3 [1, 8]	0.218
NIHSS at 3 months	1 [0, 6]	2 [1, 3]	0.079
mRS at 3 months	2 [1, 3]	2 [1, 3]	0.079
Good functional outcome at 3 months (%)	60.7	50.6	0.010
Intravenous thrombolysis (%)	22.2	33.6	0.001
Intraarterial thrombolysis (%)	-	7.0	
Etiological stroke subtypes			0.415
Atherothrombotic (%)	16.8	17.5	
Cardioembolic (%)	38.2	36.9	
Lacunar (%)	13.7	17.5	
Undetermined (%)	31.8	28.1	

Table S2. Logistic regression analysis between proBrain Natriuretic Peptide (proBNP) ≥340 pg/mL and good functional outcome at 3 months.

	Crude OR (CI 95%), p	Adjusted OR * (CI 95%), p
Cohort I and II		
All patients	1.71 (1.28 - 2.30), <0.0001	1.79 (1.15 - 2.64), 0.043
Cardioembolic strokes	0.24 (0.12 - 0.47), <0.0001	0.26 (0.11 - 0.52), <0.0001
Non-cardioembolic strokes	4.91 (3.37 - 7.15), <0.0001	3.61 (2.06 - 6.16), <0.0001

* Adjusted by age, National Institutes of Health Stroke Scale (NIHSS) at admission, body temperature at admission, systolic and diastolic blood pressure at admission, glycemia at admission and thrombolysis.

Figure S1. Improvement of neurological deficits within the first 48 hours (**A**) and 3 months (**B**). Black squares represent all patients with cardioembolic and non cardioembolic stroke. National Institutes of Health Stroke Scale (NIHSS); proBrain Natriuretic Peptide (proBNP).