Serum OPN levels are up regulated and predict disability after an ischemic stroke

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Abstract

Background: After an acute ischemic stroke (AIS), several inflammatory biomarkers have been investigated, but their predictive role on functional recovery remains to be validated. Here, we investigated the prognostic relevance of biomarkers related to atherosclerotic plaque calcification, such as osteopontin (OPN), osteoprotegerin (OPG) and the receptor activator of nuclear factor kappa-B ligand (RANKL) in a cohort of AIS patients (n=90) during 90-day follow up.

Materials and methods: Radiological and clinical examinations as well as blood sampling were performed at admission and at day 1, 7 and 90 from the event. Validated scores (such as modified Rankin Score [mRS] and the National Institutes of Health Stroke Scale [NIHSS]) were used to assess post-stroke outcome. Serum levels of OPN, OPG and RANKL were measured by colorimetric enzyme-linked immunosorbent assay (ELISA).

Results: When compared to the admission, OPN serum levels increased at day 7. Serum OPN levels at this time point were positively correlated with both ischemic lesion volume and NIHSS at day 7 and 90. A cut-off of 30.53 ng/mL was identified for serum OPN by receiver operator curve (ROC) analysis. Adjusted logistic regression showed that serum OPN levels at day 7 predicted worse mRS at day 90 (OR 4.13 [95% CI 1.64-10.36]; p=0.002) and NIHSS (1.49 [95% CI 1.16-1.99]; p=0.007), independently of age, gender, hypertension and thrombolysis.

Conclusion: Serum levels of OPN, but not OPG and RANKL peaked at day 7 after AIS and predicted worse neurological scores. Therefore, OPN might have a pathophysiological and clinical relevance after AIS.

Key words: Osteopontin, osteoprotegerin, RANKL, ischemic stroke.

Introduction

Osteopontin (OPN), osteoprotegerin (OPG) and the receptor activator of nuclear factor kappa-B ligand (RANKL) have been traditionally investigated in the field of bone turnover and metabolism [1, 2]. In the last decade, these molecules were also demonstrated to play a relevant role in vascular remodelling, atherogenesis and plaque calcification [3]. OPN is an aspartic acid-rich N-linked glycosylated protein with marked pro-inflammatory activities, as shown by disease mouse models of transgenic overexpression and gene deletion [4]. This molecule can be secreted by macrophages and enhances the expression of Th1 cytokines and matrix degrading enzymes [5]. On the other hand, the cytokine RANKL, known as a promoter of osteoclast generation and then bone reabsorption [6], was also shown to enhance leukocyte recruitment by up regulating the expression of adhesion molecules on endothelial cells as well as by promoting the release and activity of matrix metalloproteinase (MMP) from vascular smooth muscle cells (VSMCs) [3]. Although functional activity of RANKL may be inhibited in both systemic circulation and within inflamed tissues by the decoy receptor OPG [7], these mediators has been recently associated with increased cardiovascular (CV) risk [8, 9]. However, the biological role of OPG remains to be clarified, especially whether OPG activity is limited to RANKL inhibition or includes direct pro-atherosclerotic effects. In acute CV diseases, such as acute ischemic stroke (AIS), clinical studies also revealed that serum OPG might predict 90-day modified Rankin scale (mRS) [10, 11] as well as global mortality at 47-month follow up after stroke [10, 11]. However, the relationship between OPN and RANKL levels was not explored in these studies. On the other hand, OPN was shown to predict worse 90-day disability in patients with ischemic stroke treated with thrombolysis [12]. The role of OPN remained controversial when applied to the more general pathophysiological context since it was suggested as a protective molecule with neuronal anti-apoptotic effects *in vitro* [13] and *in vivo* [14]. Therefore, considering this limited knowledge about both the

pathophysiological relevance and the dynamics of these serum bone-related biomarkers, we investigated their circulating expression at admission and after 1, 7, and 90 days following AIS in a cohort of 90 patients. The potential correlations between serum biomarkers and clinical and radiological scores during the first 90-day follow up were explored.

Methods

Patients and clinical assessment

We performed a prospective cohort study by recruiting consecutive patients admitted for first AIS at Neurology Department of Ferrara University Hospital from April 2009 to December 2011. AIS was defined according to American Heart Association ischemic stroke as an acute-onset focal neurological deficit combined with neuroimaging evidence of cerebral infarction [15]. During the study period, 188 AIS patients were admitted to our Institution. Of these, 98 were excluded due to their inability to complete the radiological and clinical protocol at baseline and follow-up (n=37), clinical instability and/or poor quality of CT acquisition due to motion artefacts (n=25), admission after 9 hours from symptom onset (n=18), and previous strokes (n=7). Therefore, 90 patients with first AIS were finally enrolled. As previously described [16], all patients were treated in accordance with recommended guidelines [16, 17] and enrolled after excluding those with previous ischaemic stroke or combination of primary haemorrhagic stroke, intracranial abscess or brain cancer, acute infection, recent (<30 days) myocardial infarction or surgery, malignancy and renal/hepatic failure. Stroke subtypes were categorized according to the TOAST (Trial of ORG 10172 in Acute Stroke Treatment) criteria in: i) large-artery atherosclerosis, ii) cardio-embolism, iii) small-vessel occlusion, iv) stroke of other determined aetiology, or v) stroke of undetermined aetiology [18]. Stroke severity was assessed at different time points (time 0, day 1, day 7 and day 90) by using the National Institutes of Health Stroke Scale (NIHSS) [19]. Additional disability assessment was

performed by using the modified Rankin scale (mRS), performed at day 90 after stroke onset. As already reported, mRS ≤2 and >2 identified good and poor outcomes, respectively [20]. The Local Ethics Committee approved this study and patients gave informed consent prior to entering in the study. The study was performed in accordance with the guidelines of the Declaration of Helsinki.

Patient follow-up and study endpoint adjudication

All patients completed the 90 days of clinical follow up. The primary end-point of this study was the prediction by serum biomarkers of 90-day disability (categorized according to the mRS score). Secondary endpoints of this study included: the prediction by serum biomarkers on stroke severity (assessed by NIHSS) and lesion size (assessed by Computed Tomography [CT]) at day 7 and 90 from AIS and changes in these parameters between day 7 and 90. The percentage changes were calculated as [(value at day 7 – value at day 90)/value at day 7] x 100. Study endpoints were independently adjudicated by two study investigators (neurologists) at Ferrara University Hospital who were blinded to the results of biochemical analyses.

Estimation of the statistical power of the study

The power study calculation was based upon available data from published cohort studies [11, 12], concerning the expected prevalence of poor long-term outcome (defined by mRS >2), which have been shown to be between 17.8% and 40.4%. Our sample size allowed detecting a large effect size (0.80) with a power of 95% and with a two-sided alpha error of 5%.

Neuroimaging

Non-contrast cranial computed tomography (NCCT) was performed by a 64-slice Lightspeed VCT (GE Medical System, Milwaukee, WI; USA) from the skull base to the vertex by using an axial technique with the following imaging parameters: 120kVp, 350mA, 512x512 matrix, 25 cm-DFOV, 4x5-mm collimation, 1 second/rotation and table speed of 15mm/rotation. All NCCT images were acquired along the orbito-meteal plane with 2.5-mm (8 images/rotation) and 5-mm (4-images/rotation) slice thickness reconstruction for posterior fossa and supratentorial region, respectively [16].

The extension of early ischemic changes (hypoattenuation, loss of the gray-white matter boundary and effacement of cortical sulci) was evaluated on NCCT at time 0 by Alberta Stroke Program Early CT Score (ASPECTS), a 10-point scale that rates the presence or absence of ischemia in 10 regions included in the middle cerebral artery territory assigning a score of 1 for normal and 0 for a region showing early ischemic signs [21]. As reported elsewhere, ischemic volume was calculated on NCCT at day 1, 7 and 90 after symptom onset with a multi-slice planimetric method by summation of the hypodense areas, manually traced on each slice in which they were detectable, multiplied by slice thickness [19]. The lesion volume obtained at 90 days was considered as the final infarct size.

Blood collection and quantification

Blood samples were collected at different time points using a butterfly to reduce membrane shear stress and then drawn in tubes to obtain serum. The first sample was collected at time 0 (in thrombolysed patients within 1 hour from the beginning of thrombolysis and within 7 hours from symptom onset; in non-thrombolysed patients: within 1 hour from admission and within 9 hours from symptom onset) and at 1, 7 and 90 days after stroke onset. In addition to biomarkers, haematology parameters and blood chemistry, including plasma glucose,

triglycerides, total cholesterol, high-density lipoprotein, and low-density lipoprotein cholesterol were measured by routine auto-analyser.

Biomarkers measurements in serum

Serum OPN (R&D Systems), OPG (R&D Systems) and RANKL (Uscn Life Science, Inc, Wuhan, China) levels were measured by colorimetric enzyme-linked immunosorbent assay (ELISA), following manufacturer's instructions. The limits of detection were 31.2 pg/mL for OPN, 62.5 pg/mL for OPG and 31.25 pg/mL for RANKL. Mean intra- and inter-assay coefficients of variation (CV) were below 8% for all markers.

Statistical analysis

Analyses were performed with IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp.). Categorical data are presented as relative and absolute frequencies, whereas continuous variables were expressed as median and interquartile range (IQR). Comparisons between groups were drawn by non-parametric Mann-Whitney *U* test (the normality assumption of the variables' distribution in both groups was violated), whereas the intragroup variations of serum biomarkers at different time points were assessed by Wilcoxon test. Spearman's rank test was performed to establish correlations between biomarkers, radiological-assessed ischemic lesion volume and NIHSS and mRS. The predictive accuracy towards a worse disability score (defined as a mRS score >2) was assessed by the receiver operator curve (ROC) analysis. The area under the curve (AUC) was given with 95% confidence interval (CI) obtained using MedCalc 12.5 (MedCalc Software, Ostend, Belgium) and the cut-off point of OPN was calculated maximizing the sensitivity in according to the Youden's index. Univariate and adjusted (for age, gender, hypertension and thrombolysis) risk analyses were performed using logistic regression models. Categorized OPN (based on the

cut-off value) and risk factors were set as the dependent variable whereas mRS at day 90 was consecutively set as the independent variable. Results are expressed as odds ratio (OR) with 95% confidence intervals (95% CI). A 2-sided p-value <0.05 was considered as statistically significant.

Results

Patients' characteristics

Baseline demographic, biochemical and clinical characteristics as well as medications of AIS patients are shown in Table 1. Median age was 67 (56-76) years and 51 patients (56.6%) were male. Among the classical risk factors for stroke, 60.0% of subjects were hypertensive, 23.3% had dyslipidaemia, 13.3% diabetes mellitus and 30.0% atrial fibrillation (Table 1). Biochemical analysis showed that AIS patients had normal levels of white blood cells (WBC), red blood cells (RBC) and platelets (Table 1). Median total cholesterol was 204 mg/dL (163-227) with a median HDL-c value of 51 mg/dL (41-61). Median value of triglycerides was 121 (86-150) (Table 1). Concerning the clinical features of stroke at admission, a large part of patients was admitted within 3 hours from the onset of symptoms (77.7%), explaining the high rate of thrombolytic treatment in this study (66.6%) (Table 1). Finally, neurological impairment (median NIHSS 10 [6-14]) was very common at admission (82.2%), in accordance with a low ASPECTS score (84.4%) (Table 1).

Serum OPN levels, but not OPG and RANKL are up regulated seven days after AIS

Serum OPN levels progressively increased at day 1, 7 as compared baseline (time 0) (Figure 1A). A significant reduction in serum OPN levels at day 90 was shown when compared to day 7, indicating a peak of OPN concentration at day 7 (Figure 1A). Conversely, OPG and RANKL did not show any significant change (Figure 1B and 1C). On the other hand, the

clinical score NIHSS and the ischemic lesion volume significantly decreased between day 7 to day 90 (Figure 1D and 1E).

Serum OPN levels at peak are directly associated with increased lesion volume and worse post-stroke neurological scores

Serum OPN levels at day 7 (corresponding to the peak level) were positively correlated with both the ischemic lesion volume (r=0.298; p=0.008) and the stroke severity score NIHSS (r=0.272; p=0.016) at the same time point (Table 2). Furthermore, these positive associations were confirmed for lesion volume, NIHSS and shown also for mRS at day 90 (Table 2). However, when the % changes between day 7 and day 90 was considered, only NIHSS remained significantly correlated with OPN levels at day 7 (r=-0.325; p=0.006) (Table 2).

In addition, ROC curve analyses indicated that OPN concentration at day 7 but not at other time points had significant prognostic accuracy to predict neurological disability (mRS score >2) with an area under the curve of 0.690 (CI 95%: 0.57-0.78; p=0.011) (Figure 2). By the Youden index, a cut-off of 30.53 ng/mL was identified as the best predictor of mRS>2 at day 90 after stroke (Figure 2). At this cut-off OPN was characterized by a sensitivity of 92% (CI 95%: 92%-100%) and a specificity of 46% (CI 95%: 33%-59%) (Figure 2). RANKL and OPG levels not found to be significant predictors of any disability score upon ROC curve analyses (data not shown).

Risk analyses showed that having OPN values above the cut-off increased the risk of poor NIHSS (≥5) and mRS (>2) at day 90 by 1.4 (OR 1.41 [95%CI 1.07-1.86]; p=0.014), and 3-fold (OR 3.37 [95%CI 1.56-7.29]; p=0.002), respectively. These results remained statistical significant also after adjustment for age, gender, hypertension, and thrombolytic treatment (Table 3). Serum levels of OPG and RANKL did not show any significant correlation with radiological or clinical parameters and scores at any time points (data not shown).

Discussion

This pilot study investigated the post-stroke time course of some inflammatory molecules, such as OPN, RANKL and OPG, previously shown as key bone-related and atherosclerotic mediators [22-24]. We showed that only OPN serum levels were altered after AIS, with a significant increase peaking at day 7 after the event. Wang and co-workers previously demonstrated a delayed up regulation of OPN expression in ischemic stroke [25]. Similarly to our findings in AIS, Suezawa and colleagues confirmed this kinetic of serum OPN expression characterized by a progressive rise, reaching the peak after 5-7 days, in a human cohort of acute myocardial infarction and reperfusion [26]. In addition, these authors provided evidence that the peak of OPN was related to a worse clinical outcome, defined as increased left ventricular volume and reduced left ventricular ejection fraction [26]. Conversely, Mendioroz and co-workers have recently emphasized the prognostic power of onset-assessed OPN in a cohort of AIS patients all treated with thrombolysis, also providing cut-off point comparable with our study (27.22 vs. 30.35 ng/mL) [12].

Our results showed a detrimental and delayed role for OPN in more general cohort of AIS patients (submitted or not to thrombolysis), without identifying the cellular sources of OPN. Despite highly speculative, some studies indicated macrophages as main source of OPN [27, 28]. On the other hand, Shin and co-workers have recently associated the spatio-temporal differences in OPN expression with the patterns of phagocyte activation [29]. However, other studies suggested that macrophage recruitment within brain might be rather a consequence of endothelial dysfunction following ischemic injury. Accordingly, higher levels of OPN were shown in injured arteries (such as in atherosclerotic plaques or reperfused ischemic lesions) compared with normal vessels [30-32]. Consistent with this hypothesis, several studies highlighted the pivotal role of oxidative stress (a well-known marker of endothelial

dysfunction) as a potent inducer of OPN synthesis [33, 34]. In addition, also a wide range of molecules (including fibroblast growth factor, transforming fibroblast growth factor-β and angiotensin II) was shown to strongly up-regulate OPN mRNA synthesis in injured endothelial cells [35] and VSMCs cells *in vitro* [36]. Considering the clinical relevance of our results, we did not investigate further the OPN source in the acute phases after AIS. Translational research approaches might be helpful to clarify this issue.

As additional direct detrimental activities on brain injury and recovery, OPN was shown to bind to extracellular matrix components, notably collagen, resulting in increased blood-brain barrier (BBB) permeability [37]. Our study demonstrated a direct correlation of serum OPN levels with severity and disability scores as well as final ischemic lesion volume. When we investigated the changes from day 7 to day 90 of infarct volume and clinical scores that are expected to improve during this post-stroke follow up [38, 39], only the association between OPN levels and clinical score NIHSS remained statistically significant. These clinical results might suggest a novel potential activity by OPN to potentially participate to post-ischemic neuronal damage, thus serving as a post-stroke injury factor.

This study also focused on other bone-related inflammatory molecules (RANKL/OPG), potentially involved in atherogenesis and plaque vulnerability [23, 40, 41]. Our results, showing that the RANKL/OPG system is not altered in humans after AIS, are in partial contrast with previous studies [10, 11, 42] suggesting a weak increase in OPG serum levels. However, these studies mainly investigated the role of RANKL/OPG axis in the dynamics underlying plaque vulnerability for AIS. Thus, our study, enrolling a cohort of patients with first AIS due to different diseases, represents an original approach investigating this system in the acute phases of ischemic brain injury, independently of atherogenesis. Differently from the study by Mendioroz and co-workers, in which the predictive power of OPN levels was

related to an atherosclerotic population submitted to thrombolysis [12], our study targeted OPN and RANKL in a more general cohort characterized by ischemic brain injury.

This study has some limitations. Firstly, this single-center study has a small size so that the overall AIS community might be not truly represented. However, the involvement of a single center allowed us to avoid potential bias related to AIS treatment. We believe that larger prospective cohort studies are needed to confirm our pilot results. Another limitation is the definition of cut-off points by a post-hoc ROC analysis. This is a methodological limitation, but we had to use this approach since no previous study tested this kind of biomarkers at this time point.

In conclusion, our study showed that serum OPN levels peaked at day 7 after stroke and were associated with worse lesion volumes, severity (NIHSS) and disability (mRS) scores at day 90. Although larger clinical trials are needed to validate its predictive power on AIS clinical outcomes, our pilot study showed novel detrimental properties of serum OPN on ischemic cerebral injury early after AIS.

Conflict of interest statement: none to be declare

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Figure legends

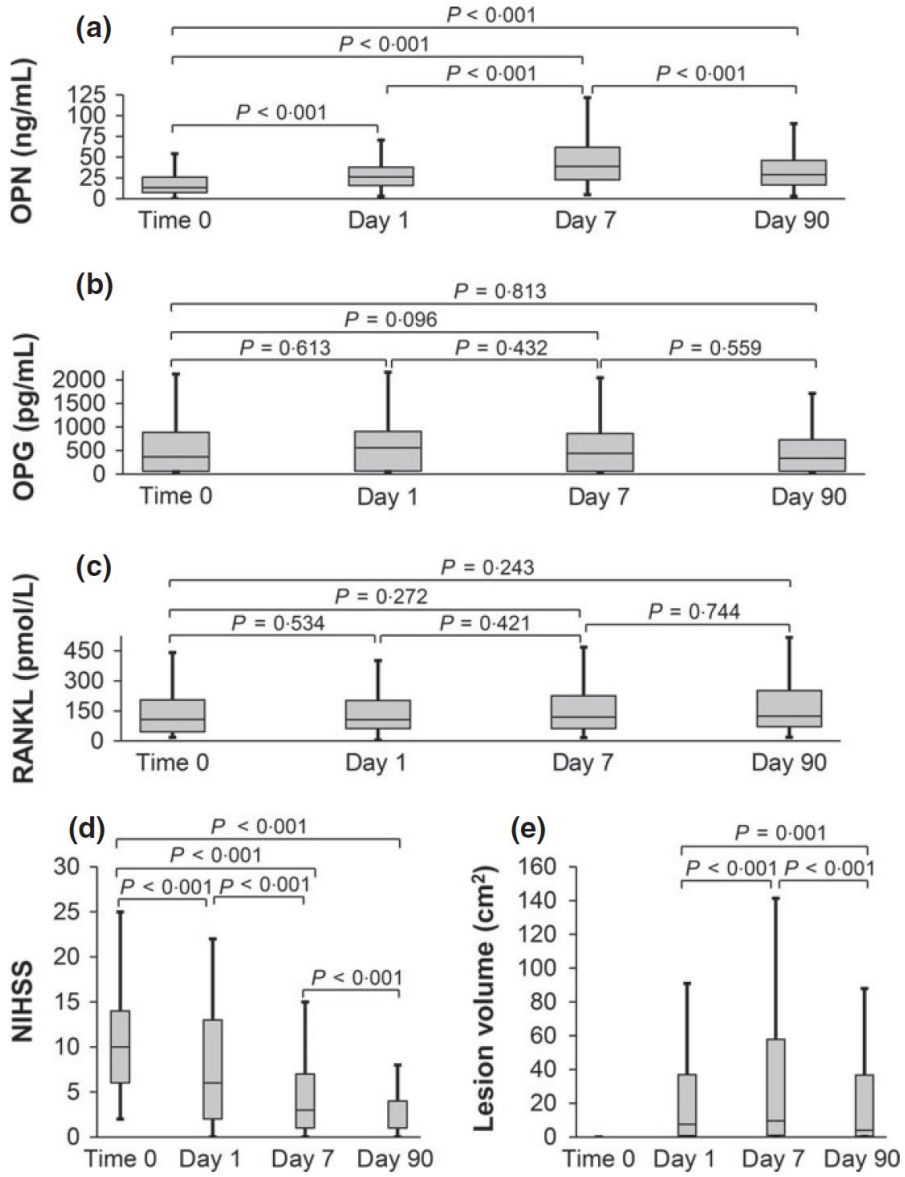

Figure 1. Serum OPN but not OPG or RANKL peaked at day 7 after stroke. Serum levels of Osteopontin (OPN) (**A**), Osteoprotegerin (OPG) (**B**) and receptor activator of nuclear factor kappa-B ligand (RANKL) (**C**) at baseline (time 0), and 1, 7 and 90 days after the acute ischemic stroke. The values of National Institute of Health Stroke Scale (NIHSS) were presented at the different time points (**D**), as well as the extension of ischaemic lesion volume assessed by computerized tomography (cm^2) (**E**). Data are expressed ad median (interquartile range).

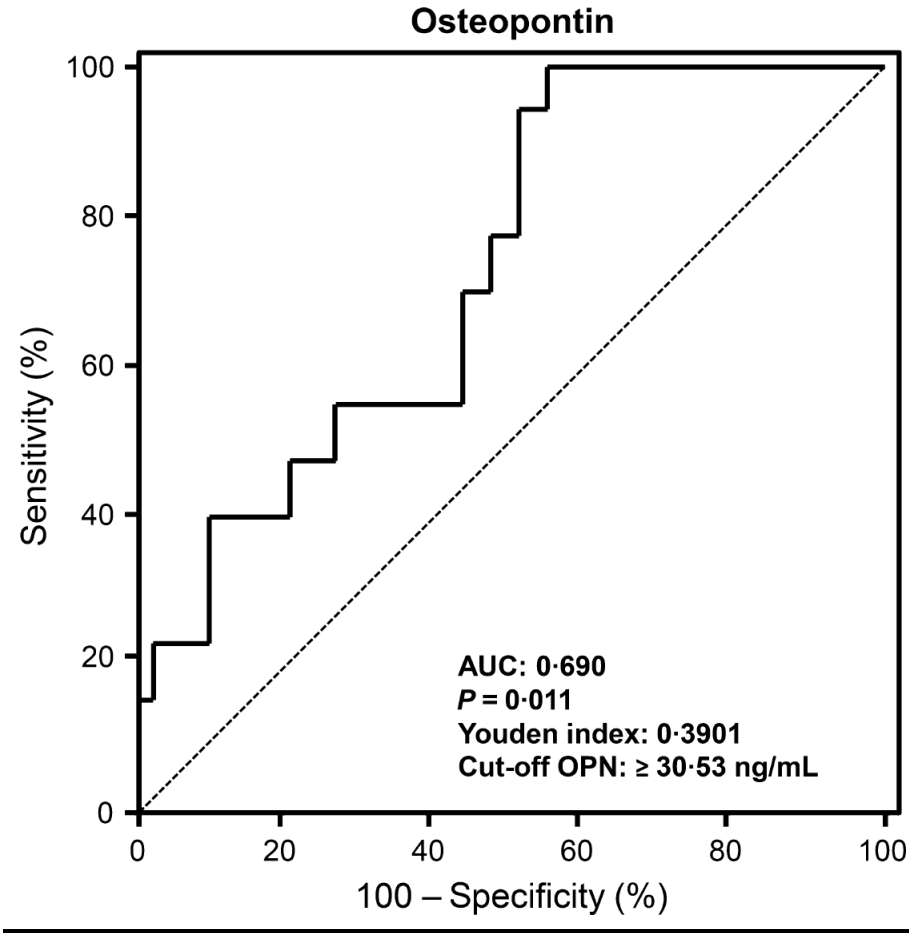

Figure 2. Receiver operation curve (ROC) analysis for OPN levels at day 7. The predicting value of serum OPN concentrations at day 7 with respect to worse disability score at day 90, defined as a modified Rankin score (mRS) >2. The figure shows the best predicting value of serum OPN (cut-off OPN: 30.53 ng/mL) according to Youden Index.

Table 1. Clinical characteristics of study population (n=90) at admission.

Demographic	
Age, years (IQR)	67 (56-76)
Male, no. (%)	51 (56.6)
Hypertension, no. (%)	54 (60.0)
Atrial fibrillation, no. (%)	27 (30.0)
Smokers, no. (%)	30 (33.3)
Previous smokers, no. (%)	11 (12.2)
Alcohol abuse, no. (%)	2 (2.2)
Diabetes, no. (%)	12 (13.3)
Dyslipidaemia, no. (%)	21 (23.3)
CAD*, no. (%)	8 (8.8)
Liver disease, no. (%)	5 (5.4)
Biochemical	
Systolic BP#, mmHg (IQR)	140 (130-160)
Diastolic BP, mmHg (IQR)	80 (75-90)
Total WBC**, no. x 10^9 (IQR)	7.74 (6.55-9.23)
Neutrophil count, no. x 10^9 (IQR)	4.72 (3.71-6.54)
Lymphocyte count, no. x 10^9 (IQR)	1.98 (1.52-2.54)
Monocyte, no. x 10^9 (IQR)	0.56 (0.43-0.68)
Platelet count, no. x 10^9 (IQR)	213 (171-241)
RBC†† count, no. x 10^{12} (IQR)	4.67 (4.39-5.04)
Total cholesterol, mg/dL (IQR)	204 (163-227)
HDL-c‡‡, mg/dL (IQR)	51 (41-61)
LDL-c§§, mg/dL (IQR)	125 (93-143)
Triglyceride, mg/dL (IQR)	121 (86-150)
Serum glycaemia, mg/dL (IQR)	111 (97-143)
INR‖‖, no. (IQR)	1.09 (1.02-1.15)
Plasma fibrinogen, mg/dL (IQR)	273 (236-309)
Clinical	
Time window to CT##	
0-3 hours, no. (%)	70 (77.7)
3-6 hours, no. (%)	18 (20)
>6 hours , no. (%)	2 (2.2)
TOAST*** classification	
Atherothrombotic, no. (%)	42 (47.7)
Cardio-embolic, no. (%)	27 (30)
Lacunar, no. (%)	19 (21.1)
Seizure at onset, no. (%)	0 (0.0)
Headache at onset, no. (%)	3 (3.2)
Previous cognitive decline, no. (%)	5 (5.4)
NIHSS†††, no. (IQR)	10 (6-14)
ASPECTS‡‡‡, no. (IQR)	10 (8-10)
Medications	
RAAS† inhibitors	36 (40.0)
ACE-I‡, no. (%)	29 (32.2)
ARBs§, no. (%)	7 (7.8)
β-blockers, no. (%)	15 (16.5)

Calcium antagonists, no. (%)	6 (6.7)
Diuretics, no. (%)	28 (31.1)
Statins, no. (%)	12 (13.3)
Antiaggregants, no. (%)	26 (28.9)
Aspirin, no. (%)	22 (24.5)
Thienopyridine, no. (%)	4 (4.4)
Oral antidiabetics, no. (%)	4 (4.3)
Insulin, no. (%)	1 (1.1)
Thrombolysis, no. (%)	60 (66.6)

Data are expressed as median (interquartile range [IQR]) or number [no.] (percentages [%]).

* CAD: coronary artery disease
† RAAS: renin-angiotensin-aldosterone system
‡ ACE-I: angiotensin converting enzyme inhibitors
§ ARBs: angiotensin receptor blockers
|| TEA: Carotid endarterectomy
BP: blood pressure
** WBC: white blood cells
†† RBC: red blood cells
‡‡ HDL-c: high density lipoprotein cholesterol
§§ LDL-c: low density lipoprotein cholesterol
|| || INR: international normalized ratio
CT: computerized tomography
*** TOAST: Trial of Org 10172 in Acute Stroke Treatment
††† NIHSS: National Institutes of Health Stroke Scale
‡‡‡ ASPECTS: Alberta Stroke Program Early CT score

Table 2. Spearman's rank correlation between serum OPN levels, stroke lesion volume, severity and disability scores.

Serum OPN* levels at day 7	*r*	*p*
day 7		
CT† lesion volume, mm^3	0.298	**0.008**
NIHSS‡	0.272	**0.016**
day90		
CT lesion volume, mm^3	0.263	**0.020**
NIHSS	0.439	**<0.001**
mRS§	0.329	**0.003**

* OPN: osteopontin
† CT: computerized tomography
‡ NIHSS: National Institutes of Health Stroke Scale
§ mRS: modified Rankin scale

Table 3. Logistic regression showing the predictive value of categorized OPN (cutoff ≥30.53 ng/mL) towards worse clinical scores at day 90.

	Univariate model			Multivariate model		
	OR*	95% CI†	*p*-value	OR	95% CI	*p*-value
mRS‡ day 90						
OPN||	3.37	1.56-7.29	**0.002**	4.13	1.64-10.36	**0.002**
Age (I vs. IV quartile)	1.07	0.64-1.79	0.794	0.61	0.26-1.45	0.271
Gender, male	0.27	0.07-1.03	0.057	0.58	0.34-1.01	0.051
Hypertension	1.40	0.43-4.53	0.565	1.66	0.84-3.26	0.138
Thrombolysis	1.45	0.42-5.03	0.550	1.08	0.62-1.87	0.773
NIHSS§ day 90						
OPN	1.41	1.07-1.86	**0.014**	1.49	1.16-1.99	**0.007**
Age (I vs. IV quartile)	0.99	0.90-1.10	0.980	0.86	0.71-1.06	0.166
Gender, male	0.88	0.79-0.98	**0.021**	0.86	0.74-0.98	**0.026**
Hypertension	1.03	0.95-1.13	0.416	1.05	0.93-1.20	0.424
Thrombolysis	1.02	0.94-1.12	0.531	1.01	0.89-1.13	0.991

* OR: odds ratio
† CI: confidence interval
‡ mRS: modified Rankin scale
|| OPN: osteopontin
§ NIHSS: National Institutes of Health Stroke Scale