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ORIGINAL ARTICLE

proMetalloproteinase-10 is associated with brain damage and clinical outcome in acute ischemic stroke

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Summary. *Background:* Matrix metalloproteinases (MMPs) mediate tissue injury during stroke but also neurovascular remodeling and we have shown that MMP-10 is involved in atherothrombosis. *Objective:* The purpose of this study was to examine the relationship between proMMP-10 and clinical outcome, assessing inflammatory and proteolytic markers, in patients with acute ischemic stroke. *Methods:* We prospectively studied 76 patients with ischemic stroke treated with tPA within the first 3 h from symptom onset, compared with 202 non-tPA-treated ischemic stroke patients and 83 asymptomatic subjects. Stroke severity was assessed with the National Institutes of Health Stroke Scale (NIHSS). Hemorrhagic transformation (HT) and severe brain edema were diagnosed by cranial CT. Good functional outcome was defined as a modified Rankin scale score ≤ 2 at 90 days. Serum levels of MMP-9, proMMP-10, TIMP-1, tumor necrosis factor-α (TNFα), interleukin-6 and cellular fibronectin were measured at admission. The effect of TNFα on endothelial proMMP-10 was assessed *in vitro*. *Results:* Serum proMMP-10 concentration in ischemic stroke patients, non-treated or treated with t-PA, which was higher than age-matched healthy subjects ($P < 0.0001$), was independently associated with higher infarct volume, severe brain edema, neurological deterioration and poor functional outcome at 3 months (all $P < 0.05$), but not with HT. proMMP-10 levels were also independently and positively associated with circulating levels of TNFα ($P < 0.0001$), which induced its endothelial expression *in vitro*, both mRNA and protein. MMP-9, however, was only associated with HT and severe edema (all $P < 0.05$). *Conclusions:* Increased serum proMMP-10 after acute ischemic stroke, associated with TNFα, is a new marker of brain damage and poor outcome.

Keywords: biological markers, inflammation, matrix metalloproteinases, stroke, tumor necrosis factor-alpha.

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Introduction

Stroke is the third leading cause of death after cardiovascular disease and cancer, and stroke survivors have an increased chance of losing functional independence [1]. Thrombolytic therapy has been shown to be beneficial for acute stroke, although conditioned by the limited time window of treatment and the risk of hemorrhagic transformation (HT) and other neurological complications [2].

Recently there has been an emphasis on the role of matrix metalloproteinases (MMPs) in stroke. MMPs are matrix-degrading enzymes with multifactorial actions strictly regulated via modulation of gene expression, compartmentalization, activation and inhibition by specific endogenous tissue inhibitors of metalloproteinases (TIMPs) [3]. Most MMPs are expressed after external induction by cytokines and growth factors in an inactive form, comprising a pro-domain upstream of the catalytic domain, and activation proceeds through its removal, catalyzed by furin-like convertases, by members of the plasminogen/plasmin cascade or by other MMPs. MMPs are rapidly up-regulated after cerebral ischemia, where they have been implicated in brain injury, blood-brain barrier (BBB) leakage, HT and also cell death [4]. Dysregulation of neurovascular MMPs during acute stroke has been proposed to underlie tissue damage by degradation of the neurovascular matrix and disruption of cell-matrix homeostasis,

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explaining some complications of the thrombolytic therapy [5]. However, MMPs also participate in beneficial neurovascular remodeling during stroke recovery [6]. Among them, MMP-9 has been proposed to play an important role because it is increased in the plasma of acute ischemic stroke patients, associated with lesion volume [7] and BBB disruption [8], and its levels may predict intracranial hemorrhagic complications after thrombolysis [9,10]. Furthermore, MMP-9 KO mice display reduced infarct size [11] and less BBB damage [12] after focal ischemia.

MMP-10, a member of the stromelysins family capable of degrading various components of the extracellular matrix and activating other MMPs, is secreted as zymogen (proMMP-10) that eventually may be activated by other proteases, such as plasmin [13]. Our group has shown that vascular MMP-10 mRNA expression can be induced by inflammatory stimuli [14,15], and that increased serum proMMP-10 is associated with subclinical atherosclerosis [16] and inflammatory conditions [17]. Besides, we have recently reported that active MMP-10 has a profibrinolytic effect *in vivo* [18]. In spite of available data suggesting that MMP-10 is involved in vascular disease, inflammation and also fibrinolysis, a role for MMP-10 has not been assessed in human ischemic stroke. Our primary aim was to investigate whether serum proMMP-10 is associated with HT, brain edema and clinical outcome in patients with acute ischemic stroke treated or not with tPA. Secondly, we compared it with other established inflammatory and proteolytic markers.

Methods

Baseline characteristics of the study population

We studied two different cohorts of patients with acute ischemic stroke: the first group included patients treated with intravenous tPA and the second included patients who did not receive tPA treatment. They were separately analyzed due to the potential effect of the interaction between tPA treatment and proMMP-10 levels on clinical outcome. Sample size was calculated using the statistical EPIDAT software (http://www.sergas.es/MostrarContidos_N3_T01.aspx?IdPaxina=62713), based on a 10% prevalence of symptomatic HT according to previous studies [19]. The minimum sample size calculated to detect this effect was made accepting an alpha level of 5% and 80% power.

The first cohort consisted of 76 patients with a first-ever acute ischemic stroke treated with intravenous tPA (0.9 mg/kg^{-1} for 1 h) within 3 h from symptom onset in the University Hospital of Santiago de Compostela. All patients were prospectively evaluated by using cranial CT and neurological and functional scales during a follow-up period of 90 days. Patients with prior disability (modified Rankin score [mRS] > 1) and known infectious, inflammatory or cancer diseases at the time of treatment were excluded.

The second cohort was selected from 283 consecutive patients with a first-ever ischemic stroke admitted to the same hospital within 12 h of symptom onset who were previously independent in their daily living activities. Patients with cancer (n = 5), chronic inflammatory (n = 7), severe hepatic (n = 4), renal (n = 3), hematological (n = 2) or infectious diseases in the 15 days prior to inclusion (n = 7) were excluded. Thirty-eight patients who received thrombolytic treatment, eight patients who did not agree to participate in the study and seven patients lost during the follow-up were also excluded; thus, a total of 202 patients were finally included.

Healthy subjects from the Clínica Universidad de Navarra, apparently free from overt vascular disease, were also included as controls as described previously [16]. Briefly, we identified a subgroup of 83 subjects with a similar age and sex distribution, from a population of 414 subjects without evidence of symptomatic cardiovascular disease according to the following criteria: (i) no history of heart disease, stroke or peripheral arterial disease; and (ii) normal electrocardiogram and chest X-ray. Other exclusion criteria were: substantial alteration of renal function (glomerular filtration < 60 mL/min^{-1}), presence of chronic inflammatory disease, and administration of anti-inflammatory medication or antithrombotic or hormone therapy in the two previous weeks. Subjects with acute infection based on clinical criteria were also excluded. Patients were considered to be hypertensive if they had systolic blood pressure > 139 mmHg and/or diastolic pressure > 89 mmHg and/or were using antihypertensive drugs.

This research was carried out in accordance with the Declaration of Helsinki of the World Medical Association (2000) and approved by the Ethics Committee of the Servizo Galego de Saúde (ischemic stroke patients), and the Clinica Universidad de Navarra (control subjects). Informed consent was obtained from each patient or their relatives after full explanation of the procedures.

Clinical variables

All patients were admitted to an acute stroke unit and treated following the European Stroke Organization guidelines [20]. Medical history recording potential vascular risk factors, blood and coagulation tests, 12-lead electrocardiogram, chest X-ray and carotid ultrasonography were performed at admission. Stroke subtype was classified according to the TOAST criteria [21], and stroke severity was assessed by a certified neurologist using the National Institute of Health Stroke Scale (NIHSS) on admission (or before tPA treatment when applicable), at 24 ± 6 h and 48 ± 6 h. Early neurological deterioration (END) was diagnosed in those patients whose NIHSS score worsened by 4 points or more within the first 48 h. Functional outcome was evaluated at 3 months using the mRS; this scale classifies patients into seven categories: 0, no symptoms at all; 1, no significant disability despite

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some symptoms, able to carry out all usual activities; 2, slight disability, unable to carry out all previous activities, but able to look after own affairs without assistance; 3, moderate disability, requiring some help, but able to walk without assistance; 4, moderately severe disability, unable to walk and attend to own bodily needs without assistance; 5, severe disability, bedridden, incontinent and requiring constant nursing care and attention; 6, dead. Good outcome was defined as mRS score ≤ 2 (independent patients) and poor outcome as mRS score > 2 (dependent or dead patients).

Neuroimaging variables

CT scans were carried out at admission and between days 4 and 7. Patients who received tPA were studied before infusion and 24–36 h after treatment. Early CT signs of infarction were evaluated on admission, and infarct volume and HT were assessed on the follow-up CT. HT was classified according to the European-Australasian Cooperative Acute Stroke Study (ECASS II) [22] and defined as symptomatic when it was associated with END. Infarct volume was calculated on the follow-up CT by using the formula $0.5 \times a \times b \times c$, where a and b are the largest perpendicular diameters and c is the number of 1-cm thick sections that contain the lesion. Severe brain edema grouped patients with grade III (shift of midline structures) or malignant brain edema (midline shift and compression of the basal cisterns associated with a rapid deterioration of consciousness).

Outcome variables

In the two cohorts of ischemic stroke patients, the analysed endpoints were infarct volume, symptomatic HT, severe brain edema, END and poor functional outcome at 3 months.

Laboratory tests

Venous blood samples, drawn from all patients at admission (before tPA treatment when applicable) were collected in Vacutainer tubes (Becton Dickinson, Madrid, Spain), allowed to clot for 60 min, centrifuged at $3000 \times g$ for 10 min, and the serum was immediately aliquoted, frozen and stored at -80 °C until analysis. Serum MMP-9, proMMP-10 and TIMP-1 were measured with commercially available quantitative enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Abingdon, UK) according to the manufacturer's instructions with a serum dilution of 1:100, 1:2 and 1:100, respectively. The ELISA kit for proMMP-10 (DM1000, R&D Systems had been formerly labeled as 'total MMP-10' and used for total MMP-10 determination in previous publications by our group [13–18,23,24]. Serum levels of IL-6 and TNFα were measured by using an immunodiagnostic IMMULITE 1000 System (Diagnostic Products Corporation, Los Angeles, CA, USA) and cellular fibronectin (cFn) was measured by ELISA (Biohit Plc, Helsinki, Finland). Determinations were performed by a trained technician blinded to clinical and neuroimaging data. Inter- and intra-assay coefficients of variation for ELISAs and immunodiagnostic assays were $< 8\%$.

Cell cultures

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cords by digestion with collagenase A (Invitrogen, Carlsbad, CA, USA) as described [13], grown until confluency, subcultured and used between passages 3 and 5. Cells were seeded in 24-well plates and at confluence were arrested overnight and stimulated with 1–5 ng/mL^{-1} human recombinant TNFα (Peprotech, Rocky Hill, NJ, USA).

Real-Time PCR

RNA was extracted from cells and reverse transcribed. Real-time PCR was performed using commercially available gene expression assays (PrimeTime qPCR Assays, Integrated DNA Technologies, Leuven, Belgium) for human MMP-10 (Hs.PT.49a.4393655.g) and human glyceraldehyde-3-phosphate dehydrogenase (Hs.PT.39a.22 214836), which was used to normalize results.

MMP-10 protein secretion

Total MMP-10 concentration was measured in conditioned media from TNFα-stimulated HUVEC with an ELISA designed for the analysis of cell culture supernates (DuoSet DY910, R&D systems). Inter- and intra-assay coefficients of variation for the ELISA were $< 6\%$.

The degree of MMP-10 activation in conditioned media was estimated by western blot. Briefly, proteins were separated by denaturing polyacrylamide gel electrophoresis (NuPAGE 4–12% Bis-Tris, Invitrogen, Carlsbad, CA, USA) and electrotransferred onto nitrocellulose membranes (iBlot, Invitrogen). Blots were incubated with a monoclonal antibody directed to the catalytic domain of MMP-10 (MAB9101, R&D Systems). Bound antibody was detected using a goat anti-mouse horseradish peroxidase-conjugated antibody (sc2005, Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and a chemiluminescent detection system (ECL Advance, GE Healthcare, Chalfont St Giles, Buckinghamshire, UK).

Statistical analysis

The results were expressed as percentages for categorical variables and as mean (SD) or median (interquartile range) for the continuous variables depending on whether their distribution was normal or not. Proportions were compared using the chi-square or Fisher's test, while

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continuous variables between groups were compared with Student's t or Mann–Whitney tests. Spearman's or Pearson's analyses were used for bivariate correlations. ANOVA was used to analyze the relationship between stroke subtypes and molecular marker levels. The influence of molecular marker levels on END, HT, severe brain edema and poor functional outcome (mRS > 2 at 3 months) was assessed by logistic regression analysis, while the influence on infarct volume was assessed by multivariable linear regression models. Each logistic regression analysis or multivariable linear regression model was adjusted for those variables with a proven biological relevance for each endpoint to avoid the possibility of finding some spurious associations. Residual plots were examined to detect potential non-linear relationships between the outcome variable and continuous independent variables. Results were expressed as adjusted odds ratios (ORs) or Beta estimate with the corresponding 95% confidence intervals (CIs). The statistical analysis was conducted using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

Results

Table 1 shows the characteristics of the study population. We prospectively studied 76 acute ischemic stroke patients treated with i.v. tPA (58% male, mean age 66.9 ± 11.3 years). Mean time to treatment from symptom onset was 140 ± 31 min (also for blood sampling). Stroke subtype was classified as atherothrombotic (20%), cardioembolic (54%), lacunar (3%) and undetermined (24%) according to the TOAST criteria. Another group of 202 acute ischemic stroke patients who did not receive thrombolytic therapy was included in the study, with similar baseline characteristics (54% men, mean age 73.5 ± 11.3 years), but longer time from onset of symptoms to blood sampling (218 ± 107 min) and different stroke subtype profile (Table 1). Finally, a third group of 83 control subjects (62% men, mean age 64.8 ± 9.5 years) was also included.

Analysis of ischemic stroke patients treated with tPA

In comparison with the age-matched control population, tPA-treated stroke patients showed much higher concentrations of proMMP-10 ($P < 0.0001$), which were not associated with either stroke subtype or early (2 h after tPA) or late (up to 24 h after tPA) recanalization. However, proMMP-10 was independently associated with infarct volume (Beta estimate, 13.08; 95% CI, 7.58–82.92), after adjusting for diastolic blood pressure, platelet count, proximal obstruction, basal NIHSS and early CT signs. An independent association was also found for MMP-9, cFn, IL-6 and TNFα (Table 2).

Patients with symptomatic hemorrhage (11%) exhibited increased proMMP-10 and MMP-9 levels ($P = 0.008$ and $P < 0.0001$, respectively), together with cFn ($P < 0.0001$) and TNFα ($P < 0.05$, Table 3). However, cFn (per each 1 μg) was the only variable independently associated with HT (OR, 1.09; 95% CI, 1.01–1.18), after adjusting for hypodensity volume, END and history of peripheral arteriopathy.

Table 1 Characteristics of the study population

	Controls (n = 83)	Stroke + thrombolysis (n = 76)	Stroke without thrombolysis (n = 202)
Demographic data			
Age, years (SD)	64.8 (9.5)	66.9 (11.3)	73.5 (11.3)
Sex, male, n (%)	51 (62%)	44 (58%)	110 (54%)
Clinical and laboratory data			
Diabetes mellitus, n (%)	14 (17%)	15 (20%)	53 (26%)
High blood pressure, n (%)	24 (29%)	42 (55%)	120 (59%)
Previous statin treatment, n (%)	21 (25%)	18 (23%)	45 (22%)
Smoking, n (%)	15 (18%)	26 (34%)	28 (14%)
Previous CV disease, n (%)	4 (5%)	12 (16%)	35 (17%)
Systolic blood pressure, mmHg	133.7 (20.2)	148.7 (23.3)	153.2 (30.3)
Diastolic blood pressure, mmHg	81.0 (9.4)	76.9 (16.2)	82.5 (17.4)
Glucose, mg/dL^{-1}	105.7 (29.7)	131.6 (43.3)	139.7 (65.1)
proMMP-10, pg/mL^{-1}	555.4 (179.1)	817.0 (525.0)	1069.5 (692.7)
Stroke diagnosis (TOAST criteria)			
Atherothrombotic, n (%)	–	15 (20%)	18 (9%)
Cardioembolic, n (%)	–	41 (54%)	71 (35%)
Lacunar, n (%)	–	2 (3%)	24 (12%)
Undetermined, n (%)	–	18 (24%)	89 (42%)

Table 2 Multivariable linear regression analysis evaluating the relationship between some molecular markers and infarct volume (dependent variable) in tPA-treated ischemic stroke patients

	Beta estimate (95% CI) unadjusted	Beta estimate (95% CI) adjusted*
MMP-9 (ng/mL^{-1})	5.52 (1.82, 9.21)	0.24 (0.01, 0.70)
proMMP-10 (pg/mL^{-1})	15.33 (1.20, 18.66)	13.08 (7.58, 82.92)
TIMP-1 (ng/mL^{-1})	0.38 (−0.16, 0.92)	0.32 (−0.36, 0.99)
Cellular fibronectin (μg/mL^{-1})	2.56 (1.00, 4.10)	2.04 (0.60, 3.47)
IL-6 (pg/mL^{-1})	10.80 (8.87, 12.73)	9.86 (7.51, 12.20)
TNF-α (pg/mL^{-1})	15.50 (8.25, 12.75)	8.99 (6.59, 11.40)

*Adjusted for diastolic blood pressure, platelet count, proximal obstruction, basal NIHSS and early CT signs.

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Table 3 Association between some molecular markers and hemorrhagic transformation, severe brain edema and early neurological deterioration (during the first 72 h from stroke onset) in tPA-treated ischemic stroke patients

	Symptomatic hemorrhagic transformation			Severe cerebral edema			Early neurological deterioration		
	No ($n = 68$)	Yes ($n = 8$)	P	No/light ($n = 66$)	Yes ($n = 10$)	P	No ($n = 64$)	Yes ($n = 12$)	P
MMP-9 (ng/mL^{-1})	775.9 ± 637.7	1687.0 ± 428.8	< 0.0001	734.4 ± 623.2	1651.9 ± 591.2	< 0.0001	788.5 ± 653.7	1434.2 ± 601.6	0.010
proMMP-10 (pg/mL^{-1})	741.3 ± 482.4	1278.4 ± 694.9	0.008	689.1 ± 348.9	1626.7 ± 512.2	< 0.0001	681.3 ± 407.2	1507.0 ± 646.9	< 0.0001
TIMP-1 (ng/mL^{-1})	237.7 ± 88.6	260.4 ± 115.2	0.454	228.3 ± 92.2	295.7 ± 93.1	0.075	243.8 ± 88.0	221.7 ± 112.8	0.667
cFn (μg/mL^{-1})	22.7 ± 13.4	47.8 ± 13.6	< 0.0001	22.9 ± 14.2	41.8 ± 14.8	< 0.0001	23.1 ± 13.9	40.7 ± 17.5	0.002
IL-6 (pg/mL^{-1})	10.5 ± 8.3	14.3 ± 7.5	0.232	10.7 ± 7.3	13.9 ± 9.1	0.091	10.2 ± 7.9	15.7 ± 9.1	0.017
TNF-α (pg/mL^{-1})	13.5 ± 7.2	20.4 ± 9.9	0.017	12.9 ± 8.1	15.7 ± 9.6	0.131	12.8 ± 6.4	22.5 ± 10.1	< 0.0001

On the other hand, as shown in Table 3, proMMP-10, MMP-9 and cFn were significantly elevated in patients with severe brain edema (13%). These molecular markers were also independently associated with severe brain edema (all $P < 0.01$) after adjusting for age, NIHSS at admission, maximal temperature within 24 h, early CT signs of infarction and hypodensity volume (Table 4).

proMMP-10, MMP-9, IL-6, cFn and TNFα were highly increased in patients with END (Table 3). However, only proMMP-10 remained independently associated in the logistic regression analysis (OR, 1.32; 95% CI, 1.01–1.72), after adjusting for diastolic blood pressure, symptomatic hemorrhage and hypodensity volume.

Likewise, proMMP-10 (1146.2 ± 521.2 vs. 466.7 ± 271.5 pg/mL^{-1}, $P < 0.0001$), IL-6 (16.2 ± 8.2 vs. 5.8 ± 4.1 pg/mL^{-1}, $P < 0.0001$), cFn (29.7 ± 16.3 vs. 21.8 ± 14.3 μg/mL^{-1}, $P = 0.045$) and TNFα (19.4 ± 7.3 vs. 9.2 ± 4.2 pg/mL^{-1}, $P < 0.0001$) were significantly higher in patients with poor functional outcome (mRS > 2) at 3 months. Logistic regression analysis showed that serum levels of proMMP-10 were independently associated with poor outcome (OR, 1.61; 95% CI, 1.09–2.17) after adjusting for age, history of atrial fibrillation, glucose levels, basal NIHSS, END, hypodensity volume and early CT signs of infarction (Table 5).

Finally, the relationship between proMMP-10 and inflammatory markers was explored and a very strong correlation was observed with TNFα ($r = 0.941$, $P < 0.0001$; Fig. 1A), being the only independent association found after multivariable linear regression analysis (Beta estimate, 59.49; 95% CI, 51.90–67.09). In contrast, MMP-9 was strongly correlated ($r = 0.634$, $P < 0.0001$; Fig. 1B) and independently associated with cFn (Beta estimate, 27.47; 95% CI, 19.34–35.61).

Analysis of ischemic stroke patients non-treated with tPA

proMMP-10 was also measured in a second group of ischemic stroke patients that did not receive tPA, in order to assess whether the proMMP-10 relationship with brain edema, neurological deficit, infarct volume and functional outcome was independent of the thrombolytic treatment. These patients showed higher

Table 4 Crude and adjusted OR of severe brain edema for molecular markers in tPA-treated ischemic stroke patients

	OR (95% CI), crude	OR (95% CI), adjusted*
proMMP 10 (per 100 pg)	1.60 (1.18–3.02)	1.67 (1.22–3.12)
MMP 9 (per 100 ng)	1.41 (1.16–2.19)	1.41 (1.13–1.95)
cFn (per 1 μg)	1.10 (1.04–1.23)	1.13 (1.10–1.17)

*Adjusted for age, NIHSS at admission, early CT signs > 33% and hypodensity volume.

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Table 5 Crude and adjusted OR of poor functional outcome (mRS > 2 at 3 months) in ischemic stroke patients

	tPA-treated		Non tPA-treated	
	OR (95% CI), crude	OR (95% CI), adjusted*	OR (95% CI), crude	OR (95% CI), adjusted*
proMMP-10 (per 100 pg)	2.03 (1.40–2.64)	1.61 (1.09–2.17)	4.02 (1.81–8.35)	3.83 (1.50–6.13)
TNF-α (per 1 pg)	1.35 (1.16–1.48)	1.21 (1.02–1.53)		

*Adjusted by age, history of atrial fibrillation, glucose levels, basal NIHSS, END, hypodensity volume and early CT signs of infarction.

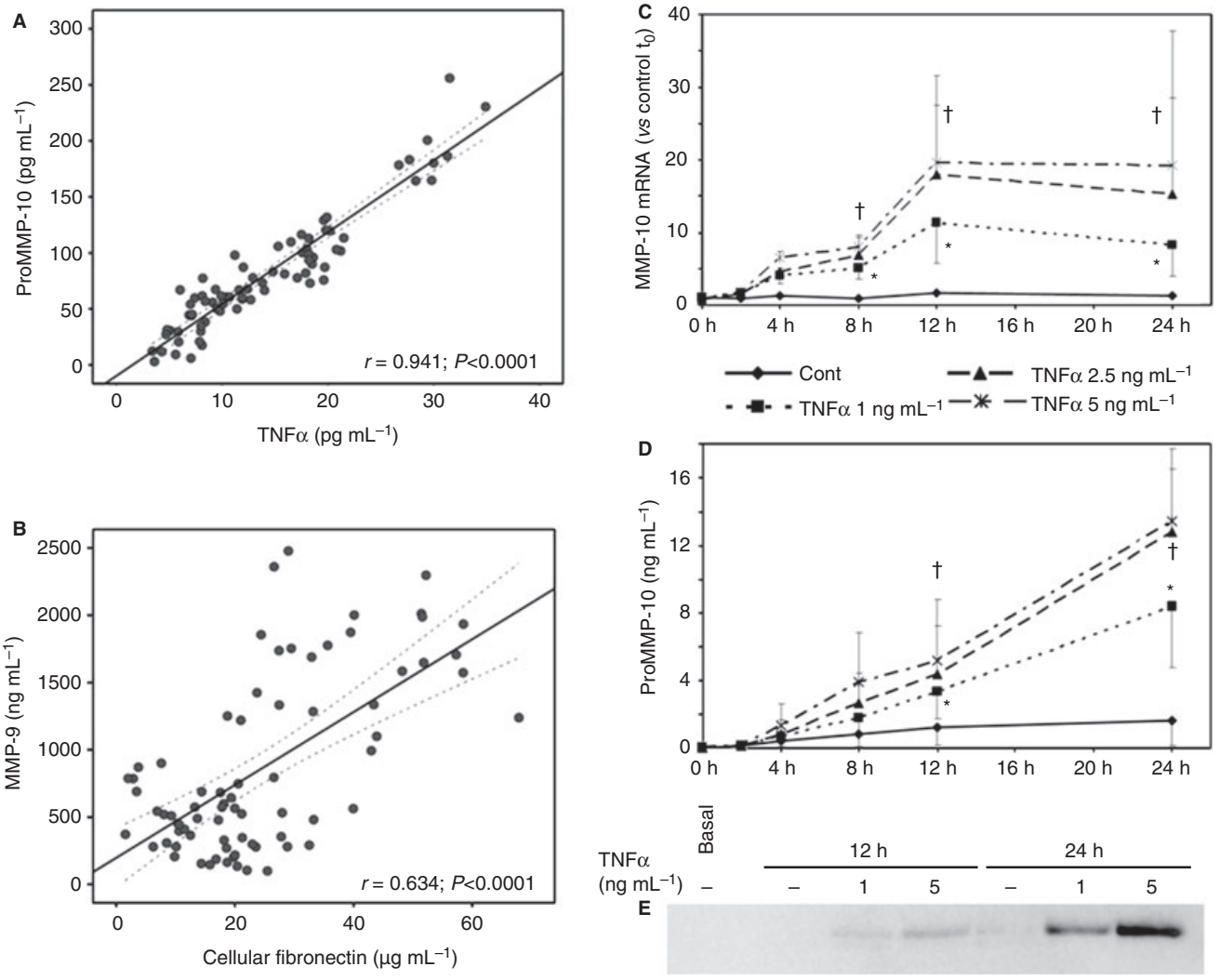

Fig. 1. MMPs are associated with inflammatory markers in tPA-treated acute ischemic stroke patients. (A) Correlation between proMMP-10 and TNFα. (B) Correlation between MMP-9 and cellular fibronectin. (C) Time course of endothelial MMP-10 mRNA expression (mean ± SD, $n = 4$) after stimulation with TNFα. (D) TNFα induces proMMP-10 protein release in human umbilical vein endothelial cell (HUVEC)-conditioned medium (mean ± SD, $n = 4$). (E) Representative western blot showing 52 kDa band corresponding to proMMP-10 in conditioned medium from TNFα-treated HUVEC. *, $P < 0.05$; †, $P < 0.01$.

proMMP-10 levels than control subjects, as expected, but also higher proMMP-10 levels than the tPA-treated group (Table 1). proMMP-10 concentration was similar in patients with different etiological stroke subtypes.

No differences in proMMP-10 levels could be observed in those patients who had HT (9%), while it was highly increased in the six patients (3%) with END (1408.1 ± 913.4 vs. 3424.1 ± 744.9 pg/mL^{-1}, $P < 0.0001$). However, proMMP-10 levels were not independently associated with END (OR, 1.12; 95% CI, 0.92–3.07) after adjusting for age, maximal temperature within the first 24 h, basal glucose levels, basal NIHSS, infarct volume and early CT signs of infarction. In contrast, proMMP-10 levels were significantly correlated with infarct size

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($r = 0.693$; $P < 0.0001$) and this association was maintained in the multivariable linear regression analysis (Beta estimate, 3.82; 95% CI, 1.63–5.13) after adjusting for gender, history of atrial fibrillation, maximal temperature within 24 h, basal glucose levels, platelets, fibrinogen, leukocytes, basal NIHSS and TOAST criteria.

On the other hand, proMMP-10 increased with the severity of brain edema (Figure S1) and was significantly elevated (2268.6 ± 1052.3 vs. 866.1 ± 353.7 pg/mL^{-1}, $P < 0.0001$) in patients with severe edema (13%), as was its inhibitor TIMP-1 (477.0 ± 266.1 vs. 358.3 ± 165.3 ng/mL^{-1}, $P = 0.008$). proMMP-10 levels (per 100 pg) were independently associated with severe brain edema (OR, 1.92; 95% CI, 1.56–3.12) after adjusting for age, maximal temperature within 24 h, glucose levels, basal NIHSS, infarct volume, early CT signs of infarction and TIMP-1 levels. No other difference or significant association could be observed for TIMP-1 and any of the clinical parameters analyzed, independently of the thrombolytic treatment.

Finally, proMMP-10 was also increased in patients with poor functional outcome at 3 months (2410.1 ± 605.9 vs. 769.4 ± 366.5, $P < 0.0001$). The higher the proMMP-10 levels, the greater the severity of mRS (Figure S2). Logistic regression analysis showed that proMMP-10 was independently associated with poor outcome (OR, 3.83; 95% CI, 1.50–6.13) (Table 5).

Relationship between TNFα and endothelial MMP-10

The tight association observed between circulating TNFα and proMMP-10 in patients subjected to thrombolysis led us to study the effect of TNFα on MMP-10 expression in human endothelial cells. As shown in Fig. 1(C), treatment of HUVEC with TNFα induced a significant time and dose-dependent increase in MMP-10 mRNA and protein. mRNA induction was already patent at 4 h, peaked at 12 h and remained at high levels for up to 24 h. mRNA up-regulation was accompanied by an increase in proMMP-10 secreted into the medium, starting at 2.2 h and reaching statistical significance by 8 h ($P < 0.05$, Fig. 1D).

Conditioned medium was subjected to western blot in order to corroborate MMP-10 up-regulation. The distinctive 52 kDa band corresponding to proMMP-10 was patent 12 h after TNFα stimulation (Fig. 1E); this was more noticeable at 24 h and even more intense with the higher dose of TNFα, while it was barely detectable in non-treated HUVEC. The 43 kDa band corresponding to the active MMP-10 could not be observed in any of the experimental conditions tested.

Discussion

We show herein that proMMP-10 levels at admission are significantly increased in ischemic stroke patients, irrespective of stroke subtype, and independently associated with infarct volume, severe edema, poor outcome and circulating TNFα, but not with hemorrhagic transformation. We have also demonstrated that TNFα induces proMMP-10 expression in endothelial cells, suggesting that the vascular wall can be a source of augmented proMMP-10.

Increased MMP expression after cerebral ischemia is well documented, both experimentally and clinically, and associated with brain tissue damage and hemorrhagic transformation (reviewed in [25]). As most of the available evidence focused on MMP-2 and -9, the overall data indicate that gelatinases play a deleterious role in the acute setting by promoting BBB degradation. In particular, increased MMP-9 concentration after stroke has been associated in humans with infarct volume, stroke severity, hemorrhagic transformation and functional outcome (reviewed in [26]). Moreover, it has been shown that MMP inhibition reduces brain damage in experimental models of stroke [27]. With this background, it was not surprising that proMMP-10 was significantly increased in patients with acute ischemic stroke subjected to thrombolysis, although gelatinases and stromelysins have different substrate specificity. This observation was in agreement with a previous proteomic protease profiling [28] and a report showing that MMP-10 was up-regulated in human brain neurons after stroke [29]. We confirmed this result in a second group of non-thrombolysed ischemic stroke patients, in whom the proMMP-10 concentration was even higher. As blood sampling was performed before treatment, differences in proMMP-10 concentration between the two cohorts could be due to their distinct characteristics, even though no differences were found among stroke subtypes, but also to the longer time from acute stroke to blood sampling in patients who did not receive tPA.

In our cohort of stroke patients receiving thrombolysis, proMMP-10 was independently associated mainly with variables related to brain damage (infarct volume, severe edema, neurological deterioration and clinical outcome) and inflammation (TNFα). In contrast, MMP-9 was more associated with HT and cerebral edema, and also with markers of vascular damage and BBB rupture (cFn), in agreement with what had been previously reported for this MMP [26,30]. Independent association of proMMP-10 with infarct volume and clinical outcome in the second group of patients further supports its role as a marker of cerebral damage, while association with neurological deterioration could not be confirmed, probably due to its low incidence in this cohort (<3%).

These results and the lack of association between circulating proMMP-10 and reperfusion could seem to be in apparent conflict with a recent report from our group demonstrating that thrombolysis with active MMP-10 is as efficient as tPA in a preclinical model of ischemic stroke, in terms of reperfusion time and infarct size, with reduced hemorrhage risk [18]. However, there is no real contradiction because active MMP-10 is required for efficient thrombolysis and increased proMMP-10 does not

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imply necessarily more active MMP-10, as we have shown *in vitro* with TNFα stimulation.

Vascular alterations in the acute phase of stroke result in the disruption of BBB permeability, causing extravasation of albumin and other high-molecular-weight compounds, which leads to edema and increased intracranial pressure. We have found that proMMP-10 is independently associated with severe edema formation, together with MMP-9 and cFn, in agreement with a previous report associating these last two markers with malignant brain edema in clinical studies [31]. However, we cannot claim a causal role for proMMP-10, because we have shown previously that proMMP-10 (up to 550 ng/mL^{-1}) does not induce endothelial cytotoxicity [18] and we lack experimental evidence linking proMMP-10 with endothelial activation and/or damage, favouring vasoconstriction and increased permeability of the BBB, which have been related to edema formation and mortality after ischemia [32].

The first cells responding to brain ischemia are glial cells, with transcription of early pro-inflammatory cytokines, such as TNFα, which may contribute to both neurotoxicity and neuroprotection either locally or by leaving the damaged tissue and entering the circulation [33]. The tight association between TNFα and proMMP-10 in peripheral blood leads us to propose that TNFα released by ischemic tissue could induce MMP-10 mRNA expression as part of the elicited response in the vascular wall. Our *in vitro* results, demonstrating that TNFα induces proMMP-10 production by endothelial cells, support this hypothesis and are in agreement with previous results from our group showing that endothelial MMP-10 mRNA expression can be up-regulated by inflammatory stimuli, such as C reactive protein,[14] thrombin [13] and CD40 ligand [15], which also belongs to the TNFα cytokine family. They are also in accordance with increased proMMP-10 levels observed in human sepsis, a systemic inflammatory condition [17]. Overall, MMP-10 up-regulation may be part of a vascular wall response to an inflammatory stimulus (TNFα) triggered by the acute ischemic event.

Limitations

We acknowledge that our research work has several limitations that should be properly addressed in future studies, mainly the limited sample size. Also, in order to have a complete scope, other MMPs should be studied, particularly those belonging to the same family (MMP-3 and -11), together with their specific inhibitors, analyzing their relationship with stroke severity and clinical outcome. We provide solid evidence associating proMMP-10 with neurological deterioration, severe edema and poor functional outcome, but cannot prove a causal role for proMMP-10 in brain damage. Further studies are warranted in order to examine proMMP-10 activation in acute stroke and elucidate whether active MMP-10 could be mechanistically involved in neuronal damage.

Conclusion

The present study shows that increased proMMP-10 concentration after acute ischemic stroke is independently associated with TNFα, brain damage, development of severe cerebral edema and poor functional outcome, and thus may represent a new marker for this disease irrespective of the thrombolytic treatment and stroke etiology. Early risk assessment in this leading cause of cardiovascular death may allow for better and earlier intervention and improved therapeutic strategies to effectively treat these patients.

Addendum

T. Sobrino, J. Orbe and J.A. Rodríguez participated in the conception, design and drafting of the manuscript, and also performed data analysis; A. Purroy participated in the analysis and interpretation of the data; E. Martínez-Vila critically revised the manuscript; J. Castillo and J.A. Páramo participated in the interpretation of data and critically revised and approved the submitted manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. proMMP-10 increases with brain edema in ischemic stroke patients without thrombolytic treatment.
Figure S2. proMMP-10 levels increase with Rankin score at 3 months in ischemic stroke patients without thrombolytic treatment.

References

1 Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, Ferguson TB, Ford E, Furie K, Gillespie C, Go A, Greenlund K, Haase N, Hailpern S, Ho PM, Howard V, Kissela B, Kittner S, Lackland D, Lisabeth L, *et al*. Heart disease and stroke statistics–2010 update: a report from the American Heart Association. *Circulation* 2010; **121**: e46–215.

2 Hacke W, Kaste M, Bluhmki E, Brozman M, Davalos A, Guidetti D, Larrue V, Lees KR, Medeghri Z, Machnig T, Schneider D, von Kummer R, Wahlgren N, Toni D. Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke. *N Engl J Med* 2008; **359**: 1317–29.

3 Tallant C, Marrero A, Gomis-Ruth FX. Matrix metalloproteinases: fold and function of their catalytic domains. *Biochim Biophys Acta* 2010; **1803**: 20–8.

4 Mun-Bryce S, Rosenberg GA. Matrix metalloproteinases in cerebrovascular disease. *J Cereb Blood Flow Metab* 1998; **18**: 1163–72.

5 Cunningham LA, Wetzel M, Rosenberg GA. Multiple roles for MMPs and TIMPs in cerebral ischemia. *Glia* 2005; **50**: 329–39.

6 Zhao BQ, Tejima E, Lo EH. Neurovascular proteases in brain injury, hemorrhage and remodeling after stroke. *Stroke* 2007; **38**: 748–52.

7 Rosell A, Alvarez-Sabin J, Arenillas JF, Rovira A, Delgado P, Fernandez-Cadenas I, Penalba A, Molina CA, Montaner J. A matrix metalloproteinase protein array reveals a strong relation between MMP-9 and MMP-13 with diffusion-weighted image lesion increase in human stroke. *Stroke* 2005; **36**: 1415–20.

8 Barr TL, Latour LL, Lee KY, Schaewe TJ, Luby M, Chang GS, El-Zammar Z, Alam S, Hallenbeck JM, Kidwell CS, Warach S. Blood-brain barrier disruption in humans is independently associated with increased matrix metalloproteinase-9. *Stroke* 2010; **41**: e123–8.

9 Montaner J, Molina CA, Monasterio J, Abilleira S, Arenillas JF, Ribo M, Quintana M, Alvarez-Sabin J. Matrix metalloproteinase-9 pretreatment level predicts intracranial hemorrhagic complications after thrombolysis in human stroke. *Circulation* 2003; **107**: 598–603.

10 Castellanos M, Sobrino T, Millan M, Garcia M, Arenillas J, Nombela F, Brea D, Perez de la Ossa N, Serena J, Vivancos J, Castillo J, Davalos A. Serum cellular fibronectin and matrix metalloproteinase-9 as screening biomarkers for the prediction of parenchymal hematoma after thrombolytic therapy in acute ischemic stroke: a multicenter confirmatory study. *Stroke* 2007; **38**: 1855–9.

11 Asahi M, Asahi K, Jung JC, del Zoppo GJ, Fini ME, Lo EH. Role for matrix metalloproteinase 9 after focal cerebral ischemia: effects of gene knockout and enzyme inhibition with BB-94. *J Cereb Blood Flow Metab* 2000; **20**: 1681–9.

12 Asahi M, Wang X, Mori T, Sumii T, Jung JC, Moskowitz MA, Fini ME, Lo EH. Effects of matrix metalloproteinase-9 gene knock-out on the proteolysis of blood-brain barrier and white matter components after cerebral ischemia. *J Neurosci* 2001; **21**: 7724–32.

13 Orbe J, Rodriguez JA, Calvayrac O, Rodriguez-Calvo R, Rodriguez C, Roncal C, Martinez de Lizarrondo S, Barrenetxe J, Reverter JC, Martinez-Gonzalez J, Paramo JA. Matrix metalloproteinase-10 is upregulated by thrombin in endothelial cells and increased in patients with enhanced thrombin generation. *Arterioscler Thromb Vasc Biol* 2009; **29**: 2109–16.

14 Montero I, Orbe J, Varo N, Beloqui O, Monreal JI, Rodriguez JA, Diez J, Libby P, Paramo JA. C-reactive protein induces matrix metalloproteinase-1 and -10 in human endothelial cells: implications for clinical and subclinical atherosclerosis. *J Am Coll Cardiol* 2006; **47**: 1369–78.

15 de Lizarrondo SM, Roncal C, Calvayrac O, Rodriguez C, Varo N, Purroy A, Lorente L, Rodriguez JA, Doeuvre L, Hervas-Stubbs S, Angles-Cano E, Paramo JA, Martinez-Gonzalez J, Orbe J. Synergistic effect of thrombin and CD40 ligand on endothelial matrix metalloproteinase-10 expression and microparticle generation in vitro and in vivo. *Arterioscler Thromb Vasc Biol* 2012; **32**: 1477–87.

16 Orbe J, Montero I, Rodriguez JA, Beloqui O, Roncal C, Paramo JA. Independent association of matrix metalloproteinase-10, cardiovascular risk factors and subclinical atherosclerosis. *J Thromb Haemost* 2007; **5**: 91–7.

17 Lorente L, Martin MM, Labarta L, Diaz C, Sole-Violan J, Blanquer J, Orbe J, Rodriguez JA, Jimenez A, Borreguero-Leon JM, Belmonte F, Medina JC, Lliminana MC, Ferrer-Aguero JM, Ferreres J, Mora ML, Lubillo S, Sanchez M, Barrios Y, Sierra A, *et al*. Matrix metalloproteinase-9, -10, and tissue inhibitor of matrix metalloproteinases-1 blood levels as biomarkers of severity and mortality in sepsis. *Crit Care* 2009; **13**: R158.

18 Orbe J, Barrenetxe J, Rodriguez JA, Vivien D, Orset C, Parks WC, Birkland TP, Serrano R, Purroy A, Martinez de Lizarrondo S, Angles-Cano E, Paramo JA. Matrix metalloproteinase-10 effectively reduces infarct size in experimental stroke by enhancing fibrinolysis via a thrombin-activatable fibrinolysis inhibitor-mediated mechanism. *Circulation* 2011; **124**: 2909–19.

19 Sobrino T, Millan M, Castellanos M, Blanco M, Brea D, Dorado L, Rodriguez-Gonzalez R, Rodriguez-Yanez M, Serena J, Leira R, Davalos A, Castillo J. Association of growth factors with arterial recanalization and clinical outcome in patients with ischemic stroke treated with tPA. *J Thromb Haemost* 2010; **8**: 1567–74.

20 European Stroke Organisation (ESO) Executive Committee; ESO Writing Committee. Guidelines for management of ischaemic stroke and transient ischaemic attack. *Cerebrovasc Dis* 2008; **25**: 457–507.

21 Adams HP Jr, Bendixen BH, Kappelle LJ, Biller J, Love BB, Gordon DL, Marsh EE 3rd. 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.

22 Larrue V, von Kummer RR, Muller A, Bluhmki E. Risk factors for severe hemorrhagic transformation in ischemic stroke patients treated with recombinant tissue plasminogen activator: a secondary analysis of the European-Australasian Acute Stroke Study (ECASS II). *Stroke* 2001; **32**: 438–41.

23 Coll B, Rodriguez JA, Craver L, Orbe J, Martinez-Alonso M, Ortiz A, Diez J, Beloqui O, Borras M, Valdivielso JM, Fernandez E, Paramo JA. Serum levels of matrix metalloproteinase-10 are associated with the severity of atherosclerosis in patients with chronic kidney disease. *Kidney Int* 2010; **78**: 1275–80.

24 Paramo JA, Beloqui O, Rodriguez JA, Diez J, Orbe J. Association between matrix metalloproteinase-10 concentration and

© 2013 International Society on Thrombosis and Haemostasis

smoking in individuals without cardiovascular disease. *Rev Esp Cardiol* 2008; **61**: 1267–73.
25 Ning M, Wang X, Lo EH. Reperfusion injury after stroke: neurovascular proteases and the blood-brain barrier. *Handb Clin Neurol* 2009; **92**: 117–36.
26 Ramos-Fernandez M, Bellolio MF, Stead LG. Matrix metalloproteinase-9 as a marker for acute ischemic stroke: a systematic review. *J Stroke Cerebrovasc Dis* 2011; **20**: 47–54.
27 Jiang X, Namura S, Nagata I. Matrix metalloproteinase inhibitor KB-R7785 attenuates brain damage resulting from permanent focal cerebral ischemia in mice. *Neurosci Lett* 2001; **305**: 41–4.
28 Ning M, Sarracino DA, Buonanno FS, Krastins B, Chou S, McMullin D, Wang X, Lopez M, Lo EH. Proteomic protease substrate profiling of tPA treatment in acute ischemic stroke patients: a step toward individualizing thrombolytic therapy at the bedside. *Transl Stroke Res* 2010; **1**: 268–75.
29 Cuadrado E, Rosell A, Penalba A, Slevin M, Alvarez-Sabin J, Ortega-Aznar A, Montaner J. Vascular MMP-9/TIMP-2 and neuronal MMP-10 up-regulation in human brain after stroke: a combined laser microdissection and protein array study. *J Proteome Res* 2009; **8**: 3191–7.
30 Morancho A, Rosell A, Garcia-Bonilla L, Montaner J. Metalloproteinase and stroke infarct size: role for anti-inflammatory treatment? *Ann N Y Acad Sci* 2010; **1207**: 123–33.
31 Serena J, Blanco M, Castellanos M, Silva Y, Vivancos J, Moro MA, Leira R, Lizasoain I, Castillo J, Davalos A. The prediction of malignant cerebral infarction by molecular brain barrier disruption markers. *Stroke* 2005; **36**: 1921–6.
32 Moldes O, Sobrino T, Millan M, Castellanos M, Perez de la Ossa N, Leira R, Serena J, Vivancos J, Davalos A, Castillo J. High serum levels of endothelin-1 predict severe cerebral edema in patients with acute ischemic stroke treated with t-PA. *Stroke* 2008; **39**: 2006–10.
33 Watters O, O'Connor JJ. A role for tumor necrosis factor-alpha in ischemia and ischemic preconditioning. *J Neuroinflammation* 2011; **8**: 87.

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