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Blood Brain Barrier Disruption in Humans is Independently Associated with Increased Matrix Metalloproteinase-9

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Abstract

Background and Purpose—Matrix metalloproteinases (MMP's) may play a role in blood brain barrier (BBB) disruption following ischemic stroke. We hypothesized that plasma concentrations of MMP-9 are associated with a marker of BBB disruption in patients evaluated for acute stroke.

Methods—Patients underwent MRI on presentation and approximately 24 hours later. The MRI marker, termed Hyperintense Acute reperfusion injuRy Marker (HARM), is gadolinium enhancement of cerebrospinal fluid (CSF) on fluid attenuated inversion recovery (FLAIR) MRI. Plasma MMP-9 and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) was measured by ELISA. Logistic regression models tested for predictors of HARM on 24 hour follow-up scans separately for MMP-9 and the MMP-9/TIMP-1 ratio.

Results—For the 41 patients enrolled diagnoses were: acute ischemic cerebrovascular syndrome 33 (80.6%), intracerebral hemorrhage 6 (14.6%), stroke mimic 1 (2.4 %) and no stroke 1 (2.4%).

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HARM was present in 17 (41.5%) patients. In model 1, HARM was associated with baseline plasma MMP-9 concentration: odds ratio (OR) = 1.01 (95% confidence interval (CI) =1.001-1.019), p=0.033. In model 2, HARM was associated with the MMP-9/TIMP-1 ratio: OR=4.94 (95% CI=1.27-19.14), p=0.021.

Conclusions—Baseline MMP-9 was a significant predictor of HARM at 24-hour follow-up, supporting the hypothesis that MMP-9 is associated with BBB disruption. If the association between MMP-9 and BBB disruption is confirmed in future studies, HARM may be a useful imaging marker to evaluate MMP-9 inhibition in ischemic stroke and other populations with BBB disruption.

Keywords

blood-brain barrier; acute cerebrovascular event; matrix metalloproteinase-9

Blood brain barrier (BBB) disruption following ischemic brain injury initiates a series of detrimental events, escalating secondary injury and the likelihood of poor outcome.[1-3] Proteolytic breakdown of the BBB vasculature increases the permeability of the barrier within hours of ischemia resulting in vasogenic edema, leukocyte infiltration, and hemorrhagic transformation (HT). The identification of factors contributing to and associated with impaired BBB integrity in humans following ischemia and reperfusion may be crucial to developing stroke therapies that can improve the safety of thrombolytics. There is evidence, in both humans and animal stroke models, that activation of matrix metalloproteinase's (MMP's), and specifically MMP-9, may contribute to proteolysis of the BBB basal lamina.[4, 5] However, a direct association between MMP-9 and the presence of imaging identified BBB disruption has yet to be demonstrated in humans.

MMP's are critical regulators of the extracellular matrix (ECM). ECM homeostasis is maintained by a balance between pro- and anti-proteolytic factors including MMP-9 and its natural inhibitor tissue inhibitor of matrix metalloproteinase-1 (TIMP-1). Because it is the balance of these pro- and anti-proteolytic factors that contributes to impairment of the BBB, the MMP-9/TIMP-1 ratio has been used to provide an in vivo assessment of the proteolytic potential of MMP-9 to degrade the ECM.[6] In stroke patients, the MMP-9/TIMP-1 ratio has been shown to have a more significant association with injury and the volume of peri-hematomal edema, than when MMP-9 and TIMP-1 are considered independently.[7] In addition, the MMP-9/TIMP-1 ratio has been identified as a significant predictor of total anterior circulation infarct (TACI) stroke subtype. [8] Therefore, the balance between MMP-9 and TIMP-1 is likely to play a role in BBB injury.[9]

BBB disruption following acute ischemic stroke can be visualized on fluid attenuated inversion recovery (FLAIR) magnetic resonance images (MRI). This phenomenon has been previously described and termed HARM (Hyperintense Acute reperfusion injuRy Marker).[1, 2] HARM is gadolinium-diethylene triamine penta-acetic acid (Gd-DTPA) enhancement of the cerebrospinal fluid (CSF) space, primarily the subarachnoid CSF space in the hemispheric sulci. Gd-DTPA does not readily cross an intact BBB; therefore its presence within the CSF space implies an impaired BBB.

Post-Gd-DTPA FLAIR identification of HARM has been confirmed in subsequent studies of stroke and multiple sclerosis, identifying its utility in clinical practice as a marker of BBB disruption. [10, 11] HARM has been observed in 33% of ischemic stroke patients imaged at 24 hours post injury and is significantly associated with reperfusion, HT, and poor clinical outcome.[1, 2] Treatment with thrombolytic agents significantly increases the prevalence of HARM in this population.[12]

Recombinant tissue plasminogen activator (rtPA) is a physiologic activator of MMPs and is the only FDA approved treatment for ischemic stroke. The administration of rtPA for the treatment of ischemic stroke can increase circulating MMP-9 both by direct and in-direct mechanisms [13] contributing to the initiation of ECM remodeling.13¯15 This process ultimately results in barrier disruptions with subsequent HT. HT following ischemic stroke has important implications for clinical outcome, however the molecular underpinnings of the process are not completely understood. Stroke severity16, age[17], and thrombolytic administration[17] have been associated with the development of HT. Elevated plasma MMP-9 has emerged as a predictor of HT[18, 19], particularly following rtPA administration [20].

BBB disruption is a primary event initiating HT, and given associations between plasma MMP-9 and the occurrence of HT, we hypothesized that plasma MMP-9 would be associated with HARM, a marker of BBB disruption. The goal of this study was to identify predictors of BBB disruption through regression models containing MMP-9 and the MMP-9/TIMP-1 ratio with previously identified covariates of HARM.

Materials and Methods

Subjects

This was a prospective study of acute stroke referrals admitted to Washington Hospital Center, DC between May 2006 and June 2007. This study was approved by both the National Institute of Neurological Disorders and Stroke and Washington Hospital Center Institutional Review Boards. Written informed consent was obtained from patients with suspected acute cerebrovascular events or their authorized representatives. Patients were included in this study if MRI and blood samples were available both at the time of presentation and approximately 24 hours later. Patients were excluded if they were less than 18 years of age, had a contraindication to MRI, or were pregnant. The time of stroke symptom onset was determined as the time the patient was last known to be free of the acute stroke symptoms. Patient evaluations and management were standardized. Thrombolytic therapy with rtPA was administered to patients who met standard eligibility criteria.

Magnetic Resonance Imaging Protocol

Patients evaluated for suspected stroke in the emergency room underwent MRI on presentation and approximately 24 hours later on a 3 Tesla clinical MRI system. The scanning protocol was standardized for sequence parameters and order of acquisition: Diffusion-weighted imaging (DWI), T2*-weighted gradient recalled echo (GRE), T2-weighted FLAIR, contrast enhanced MR angiography (MRA), and perfusion-weighted imaging (PWI), performed in that order. Gd-DTPA was administered on the acute and follow-up scans at a dosing of 0.1 mmol/kg for contrast enhanced MRA of the head and neck and for perfusion-weighted imaging. Maps of mean transit time (MTT) obtained at baseline were viewed along side of acute DWI, blinded to all other series and time points, and evaluated for the existence of a perfusion deficit. For those patients found to have a perfusion deficit, follow-up MTT maps were reviewed to determine whether reperfusion had occurred with respect to baseline, by consensus. A visually obvious improvement in the MTT maps, typically representing a volume difference of at least 30%, was considered evidence of reperfusion.

HARM Assessment

Baseline and 24-hour follow-up images were presented to expert readers in a randomized order, absent of patient identifiers and clinical outcome. All image interpretations were by visual inspection and performed jointly by consensus with a third blinded investigator resolving disagreement. All FLAIR images were reviewed sequentially in time to allow for the pre- and post-Gd-DTPA comparison between baselineand 24-hour scans. Presence of HARM (BBB

disruption) was identified as positive if the CSF intensity in the sulci, ventricles, background, or vitreous appeared hyperintense and continuous across greater than 10 slices. The 24-hour scan was used as the criterion for analysis to allow for the maximum accumulation of enhancement.

MMP-9 & TIMP-1 Determination

Peripheral blood samples were collected during the baseline work up and approximately 24 hours later in sodium heparin sampling tubes, placed immediately on ice, and centrifuged within 15 minutes at 2000g for 10 minutes. The plasma was transferred to cryovials for storage at −80°C until analysis.

Plasma MMP-9 and TIMP-1 concentrations were determined by a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) obtained from R&D Systems, Minneapolis, MN. The MMP-9 ELISA is designed to measure total MMP-9 (92 kDa pro- and 82 kDa active forms). Detectable ranges for MMP-9 and TIMP-1 in healthy controls were 13.2-105ng/ml and 39-279ng/ml respectively. Patient samples were prepared according to manufacturer recommendations, and freeze thaw cycles were avoided. All ELISA microplates were read by the SPECTRAmax Ms ROM v2.00b73. Known standards were included on all plates and unknown samples were assayed in duplicate. Concentration values with coefficients of variation (CV) > 10% were re-assayed. MMP-9/TIMP-1 ratios were calculated to provide a representation of the net proteolytic activity present in the plasma sample of each patient.

Statistical Analysis

Descriptive and frequency analysis was obtained for all data. Because MMP-9 and TIMP-1 were not normally distributed, data for the biomarkers are presented as median with range. Statistical significance between groups for univariate analysis was determined by Chi-square for categorical variables, Student's t-test or ANOVA for continuous variables, and Mann-Whitney tests as appropriate, using a criterion of $p < 0.05$. Binary logistic regression models were tested to determine whether baseline MMP-9 or MMP-9/TIMP-1 ratio were predictors of HARM. The models were entered as forward conditional with an entry and exit criterion of 0.15, to test the relationship between HARM at 24 hours and the following covariates: baseline MMP-9 or baseline MMP-9/TIMP-1 ratio, baseline DWI lesion volume, age, baseline NIHSS, hours from onset to baseline gadolinium injection, rtPA administration (yes/no), whether or not rtPA was given before the baseline blood sampling (yes/no), and reperfusion (yes/no). All statistical analyses were performed using SPSS v15.

Results

Patient Characteristics

A total of 41 patients met inclusion criteria. Diagnoses included acute ischemic cerebrovascular syndrome 33 (80.6%), intracerebral hemorrhage 6 (14.6%), stroke mimic 1 (2.4 %) and no stroke 1 (2.4%). Results are presented as mean ± standard deviation (SD) and median. Mean age of the sample was 62 ± 13.9 years (range 34 to 85 years). Median NIHSS score on admission was 4 (range 0 to 27). Median time from onset to first blood draw was 9.8 hours, to baseline imaging was 5 hours, and from baseline blood draw to follow up MRI was 23 hours. Eight patients were treated with intravenous (IV) rtPA within 3 hours of symptom onset and one intra-arterial (IA) rtPA patient was treated at 3 hours and 45 minutes following symptom onset. Of the 33 stroke patients, 30 patients had readable MTT scans at baseline and n=20 had a perfusion deficit. Of these 20 patients, 18 patients had readable MTT scans at follow-up and n=12 patients reperfused.

HARM Characteristics

Of the 41 patients, 17 (41.5%) had HARM present on the 24 hour follow-up scan. Eight (47%) of those patients had hemorrhage present on the 24 hour GRE (χ^2=2.2; p=0.14). Two of the eight patients with a positive GRE were diagnosed with primary hemorrhages. Refer to Figure 1 for examples of HARM.

Table 1 provides the univariate associations between clinical variables and the presence of HARM. Acute severity of injury as measured by the baseline NIHSS (p=0.015), baseline DWI lesion volume (p=0.036), and age (p<0.001), were significantly different between patients with and without HARM at 24 hours. Patients who were older, with larger lesion volumes, and higher NIHSS scores were more likely to have HARM. For the 33 ischemic stroke patients, these relationships with HARM also remained significantly associated with age (p<0.001), baseline NIHSS (p=0.055), and rtPA administration (p=0.001). Eight of nine patients treated with rtPA presented with HARM on the 24 hour scan (χ^2=10.7; p=0.001).

There was no relationship between HARM and reperfusion at follow up; however there was a trend for an association with a perfusion deficit on the baseline MRI and the presence of HARM on the follow up MRI (χ^2=5.66; p=0.059).

Biomarkers and Relationship to HARM

Median plasma MMP-9 concentration was 36.8 ng/ml (range 0-714.03 ng/ml) and median TIMP-1 concentration was 129.3 ng/ml (range 0-539.52 ng/ml) on the baseline blood draw for the entire sample. MMP-9 values were significantly lower at 24 hours compared to baseline (Wilcoxon Z=−2.216; p=0.027) and TIMP-1 values were significantly higher at 24 hours compared to baseline (Wilcoxon Z=−2.757; p=0.006). Baseline plasma MMP-9 concentrations were not significantly different between patients with and without HARM at 24 hours (Mann-Whitney U=183; p=0.58). However, baseline TIMP-1 concentrations were slightly higher for those patients who developed HARM (Mann-Whitney U=126; p=0.04). When the sample was dichotomized by groups based on acute ischemic cerebrovascular syndrome (AICS) criteria [21], definite AICS patients also had higher baseline TIMP-1 values associated with the presence of HARM at 24 hours (Mann-Whitney U=74; p=0.03)

On univariate analysis, the baseline MMP-9/TIMP-1 ratio was not statistically associated with the presence of HARM for either the entire population or definite AICS patients alone; however there was a trend for higher MMP-9/TIMP-1 ratios in patients with HARM. There was no relationship between MMP-9, TIMP-1, or the MMP-9/TIMP-1 ratio with the presence of a perfusion deficit on the baseline MRI or reperfusion at follow up.

A logistic regression analysis was performed to identify whether MMP-9 or the MMP-9/TIMP-1 ratio were predictors of HARM, controlling for previously known covariates and those shown to be significant in univariate analysis. Table 2 provides the regression coefficients, odds ratios, 95% confidence intervals for the odds ratios, and p values for the significant variables in each model. In model one, HARM was associated with baseline MMP-9 (p=0.03), age (p=0.005), and rtPA administration (p=0.019). In model 2, HARM on the 24 hour follow-up scan was associated with baseline MMP-9/TIMP-1 ratio (p=0.021), age (p=0.005), and rtPA administration (p=0.021).

Discussion

This study demonstrates a relationship between plasma MMP-9 and the presence of HARM, a neuroimaging marker of BBB disruption in humans. This finding is consistent with preclinical animal studies[9, 22, 23 18, 24] and supports prior clinical data suggesting relationships between HARM and BBB disruption [1, 2] and between MMP-9 and BBB disruption [18, 19, 24-26 27]. If

further validation of this finding is achieved in a larger cohort of patients, it may be clinically significant to monitor plasma MMP-9 for the early detection of BBB impairment following stroke. In addition, HARM may be a useful imaging marker to evaluate MMP-9 inhibition for the treatment of BBB disruption in stroke and other neurological diseases.

MMP-9 expression is the result of activated leukocytes (particularly neutrophils),[23] and results in IL-1beta activation28 and initiation of the inflammatory cascade29, further contributing to BBB impairment. MMPs are closely related to endogenous tPA concentrations 22, [30-32] such that endogenous tPA enhances MMP-9 expression and plays a role in MMP-9/heparin-induced HT.[14] Early inhibition of MMP-9 and MMP-9 gene knockout mice models consistently demonstrate decreased infarct volumes and attenuation of BBB disruption and inflammation. 28, 33, 34 However the timing of inhibition is critical and late inhibition of MMP-9 can be detrimental; suggesting a role for MMP-9 in neurovascular remodeling and recovery following ischemic brain injury.[35] The timing associated with this switch from deleterious to beneficial is relatively unstudied, which complicates the use of MMP-9 inhibition clinically. However, there is a scientific case for the use of MMP-9 inhibition to offset the negative effects associated with rtPA therapy (e.g. HT). Published data suggest that early MMP-9 inhibition (possibly within the same time window as that accepted for rtPA therapy) in stroke may be beneficial, especially when used in combination with thrombolytic therapy to attenuate inflammation and BBB disruption.

In stroke patients, MMP-9 has been shown to have an association with NIHSS[36] and severity of injury [8, 37], lesion volume growth [25, 38], rtPA administration [39, 40], and other peripheral biomarker proteins (cellular fibronectin19, F2IP's41, and IL-642). Overwhelming evidence identifies a risk between elevated baseline MMP-9 concentrations and subsequent HT and parenchymal hematoma (PH) for ischemic stroke patients.18-20, 26 Similarly, we found a significant association between the baseline MMP-9 and the NIHSS for patients with HARM (p=0.025). Postmortem studies validate that MMP-9 is increased in infarct and peri-infarct human brain tissue of stroke patients.[24, 27, 43] The potential of peripheral blood measurements of MMP-9 as a biomarker of proteolytic activity within the cerebral environment is promising.

Given that the median time of baseline blood sampling was about 10 hours from onset of symptoms (~18 hours for the mean of the sample) it is possible that the initial increase of MMP-9 in response to the cerebrovascular event was missed with the later blood draw. This would explain why we can identify a significant univariate relationship between TIMP-1 and HARM and not MMP-9 and HARM. TIMP-1 levels are increased acutely in response to an increase in MMP-9, and TIMP-1 levels continue to rise at 24 hours.[39] When the baseline MMP-9 concentration is entered into a multivariate model that controls for the effects of other significant covariates, the association between MMP-9 and HARM is revealed.

There are other potential limitations to this study. It is well known that plasma MMP-9 may be elevated in patients with hypertension[44], dyslipidemia[45], inflammatory/infectious processes46 diabetes44 and in patients who smoke 47. Atherosclerotic disease involves matrix remodeling and MMP-9 activation for each of the above named disorders.[48] Patients in this study presented with differing degrees of comorbidities and were not adequately screened for the presence of heart disease or infectious processes. However the patient groups were not significantly different from one another based on past medical history. In addition, four out of the nine (44.4%) patients who received rtPA had their blood draw after rtPA administration, which could lead to a false increase in plasma MMP-9. Some studies report results of plasma MMP-9 isolated from whole blood drawn after rtPA administration.[41] Because this was found to be significant in our population it was used as a covariate in the logistic regression analysis. Lastly, our patient population was small, and replication of the results on a larger independent sample is required.

Disruption of the BBB is a serious complication of ischemic stroke that may contribute to subsequent edema, initiation of the inflammatory cascade, HT and poor clinical outcome. Identification of the factors resulting in BBB disruption may ultimately aid in therapeutic drug discovery for the treatment of ischemic stroke. Data presented here are among the first to validate preclinical studies suggesting a significant relationship between MMP-9 and the presence of BBB disruption in humans. The significant associations presented here between MMP-9 and the MMP-9/TIMP-1 ratio with HARM provides further evidence of the relationship between MMP-9 and blood brain barrier disruption. It remains to be determined whether MMP-9 is an active player in blood brain barrier disruption or is a marker of the severity of the ongoing damage to the blood brain barrier following ischemic stroke. Future studies should be designed to elucidate whether MMP-9 plays a causative role in BBB disruption following stroke.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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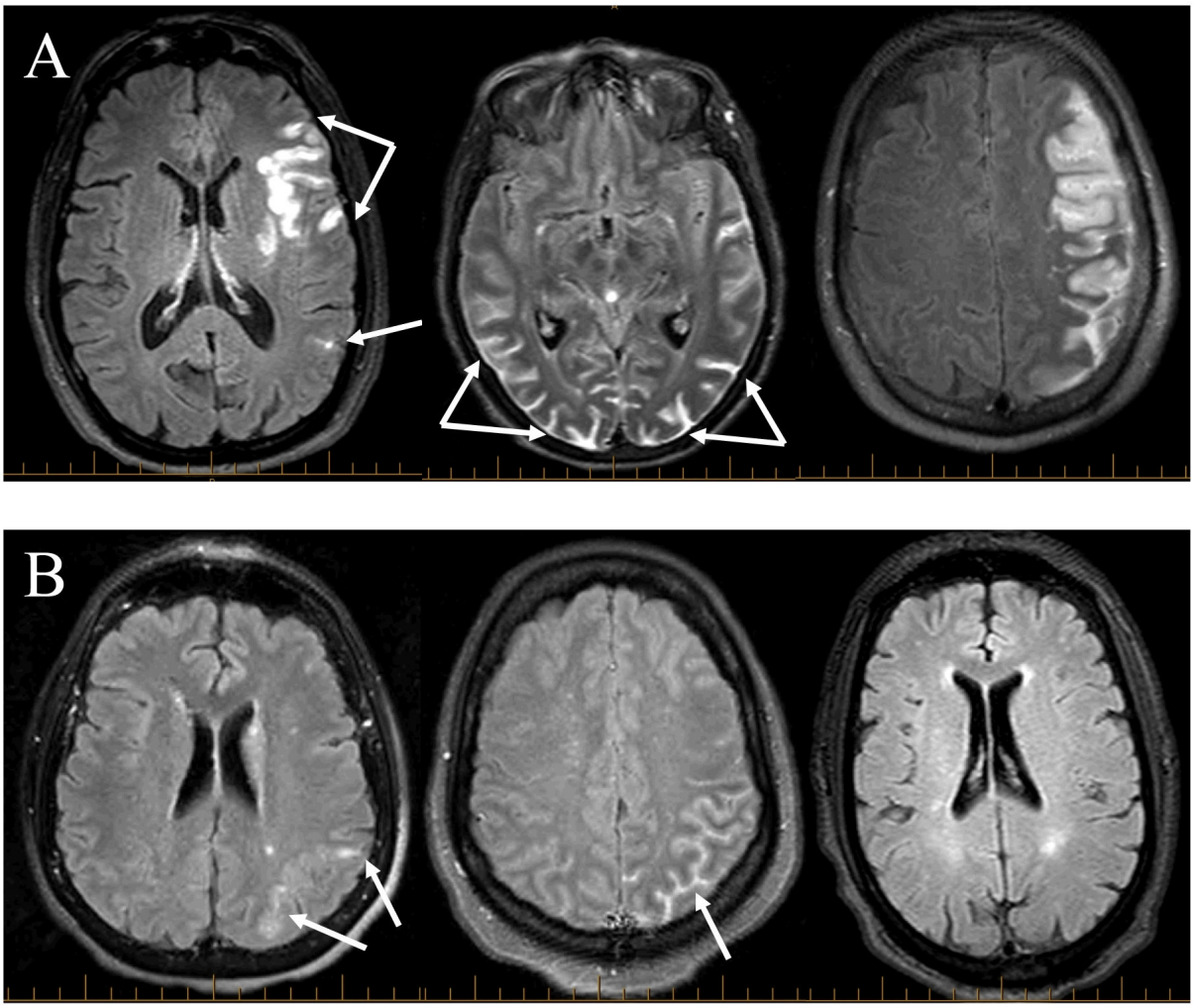

Figure 1.
Representations of HARM: **A:** Three individual cases of HARM. From Left: In the first two images areas of HARM are demonstrated by arrows. In the last image, HARM can be seen throughout the left hemisphere.
B: Three individual cases of No HARM. From Left: The firs two images have areas of hyperintensity within one or two slices of MRI image, and are therefore read as “No HARM”. The last image contains no areas of hyperintensity.

Table 1

Univariate associations between clinical variables and the presence of HARM

24 Hour HARM

	Present (n=17)	Absent (n=24)	p
Ischemic Stroke	14 (82.4%)	19 (79.2%)	0.80
Male n (%)	9 (52.9%)	14 (58.3%)	0.73
Age, years	71.1±12.2	55.42±11.2	<0.001*
DWI Lesion (cc)	35.5±46.2	11.9±22.5	0.04*
Baseline NIHSS, median	10	3	0.015 *a*
Onset to contrast, hours, median	3:00	7:30	0.80
rtPA administration	8 (47.1%)	1 (4.2%)	0.001 *b* *
WBC count	7.8±2.1	7.9±3.3	0.97
Hypertension	13 (76.5%)	15 (62.5%)	0.34
Glucose mg/dL	137.1±53.6	149.1±74.8	0.58
Baseline MMP-9 ng/ml	42.1 (0-705.8)	35.5 (0-384.9)	0.58
Baseline TIMP-1 ng/ml	139.2 (0-526.5)	119.7 (0-324.6)	0.04*
Baseline MMP-9/TIMP-1	0.23 (0-4.8)	0.31 (0-1.7)	0.53
HT at 24hr follow up	8 (47.1%)	6 (25%)	0.14

Biomarkers are presented as median (range)

HARM=Hyperintense Acute reperfusion injuRy Marker; DWI=diffusion weighted imaging; NIHSS=National Institutes of Health Stroke Scale Score; rtPA=recombinant tissue Plasminogen Activator; WBC=white blood cell count; MMP-9= matrix metalloproteinase-9; TIMP-1=tissue inhibitor of matrix metalloproteinase-1; HT=hemorrhagic transformation

a Fisher's exact test with NIHSS as Mild (0-3), Moderate (4-15) , or Severe (≥16)

b Fisher's exact test with rtPA administration as "Yes" or "No"

* significant at $p < 0.05$

Table 2

Results of Binary Logistic Regression Models for Prediction of HARM

Model 1

Predictor	b	Wald	Odds Ratio (OR)	95% CI for OR	p
Baseline MMP-9	0.010	4.548	1.010	1.001-1.019	0.033
Age	0.188	8.041	1.206	1.060-1.373	0.005
IV or IA rtPA	4.034	5.502	56.49	1.941-1644.399	0.019
Onset to Contrast	0.159	3.229	1.172	0.986-1.393	0.072

Model 2

Predictor	b	Wald	Odds Ratio (OR)	95% CI for OR	p
Baseline MMP-9/ TIMP-1 ratio	1.597	5.332	4.936	1.273-19.136	0.021
Age	0.224	7.912	1.252	1.070-1.464	0.005
IV or IA rtPA	3.787	5.327	44.106	1.77-1099.025	0.021
Onset to Contrast	0.173	3.626	1.189	0.995-1.421	0.057

Note. MMP-9 (matrix metalloproteinase-9); IV (intravenous); IA (intra-arterial); rtPA (tissue plasminogen activator)