NULL HYPOTHESIS

Analysis of the association of MPO and MMP-9 with stroke severity and outcome

Cohort study

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Neurology® 2020;95:e97-e108. doi:10.1212/WNL.0000000000009179

Abstract

Objective

In acute cerebral ischemia, circulating neutrophil count and neutrophil-to-lymphocyte ratio (NLR) are positively associated with stroke severity and worse outcomes. Mediators of this effect are unknown. We aimed to investigate (1) the relationship between plasma matrix metalloproteinase-9 (MMP-9) and myeloperoxidase (MPO) concentrations with stroke severity and outcome and (2) MMP-9 and MPO release after ex vivo stimulation of neutrophils by recombinant tissue plasminogen activator (rtPA).

Methods

We analyzed data collected in 255 patients with supratentorial cerebral infarcts recruited within 48 hours of symptoms onset irrespective of rtPA treatment. The endpoints were excellent outcome (modified Rankin Scale score 0–1), symptomatic intracerebral hemorrhage (European Cooperative Acute Stroke Study–II definition), and death at 3 months. The role of rtPA treatment on peripheral neutrophil degranulation was investigated in 18 patients within 4.5 hours and after 72 hours.

Results

Neutrophil counts, NLR, and MPO plasma concentrations, but not MMP-9, were positively correlated with stroke severity. Higher neutrophil counts and NLR were independently associated with worse outcomes and higher mortality rates at month 3. Higher MPO plasma concentrations, but not MMP-9, were associated with worse outcome. Neutrophil-derived MMP-9, after ex vivo rtPA stimulation, but not MPO, were higher after 72 hours in patients treated by IV rtPA but not associated with hemorrhagic transformation.

Conclusions

Neutrophil counts, NLR, and MPO plasma concentrations are associated with worse outcome in patients with acute cerebral ischemia, in contrast to MMP-9. Further investigations are needed to deepen our knowledge on MPO's role in the deleterious effect of neutrophils because it could represent a potential therapeutic target.

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Go to Neurology.org/N for full disclosures. Funding information and disclosures deemed relevant by the authors, if any, are provided at the end of the article.

This Null Hypothesis article is published as part of a collaborative effort, between Neurology and CBMRT.

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Glossary

HT = hemorrhagic transformation; **IS** = ischemic stroke; **MMP-9** = matrix metalloproteinase-9; **MPO** = myeloperoxidase; **mRS** = modified Rankin Scale; **NIHSS** = NIH Stroke Scale; **NLR** = neutrophil-to-lymphocyte ratio; **RPMI** = Roswell Park Memorial Institute; **rtPA** = recombinant tissue plasminogen activator; **sICH** = symptomatic intracerebral hemorrhage.

A higher circulating neutrophil count in ischemic stroke (IS) is associated with higher stroke severity, larger infarct size, increased risk of hemorrhagic transformation (HT), and worse outcomes,[1,2] regardless of IV recombinant tissue plasminogen activator (rtPA) administration.[3,4] Some observations suggest that plasma matrix metalloproteinase-9 (MMP-9) can be responsible for the deleterious effect of neutrophils in acute IS: (1) there is an increase in leukocyte MMP-9 mRNA within the first 3 hours after stroke onset[5]; (2) the MMP-9 plasma concentration is higher in patients with IS compared to controls[6]; and (3) higher MMP-9 plasma concentrations are associated with larger infarct volume,[7] worse stroke severity,[7,8] worse functional outcomes,[8–10] and increased risk of HT, irrespective of treatment with IV rtPA.[9,11–15] Because MMP-9 is released from neutrophils, it is considered the main mediator of this effect.[16]

Myeloperoxidase (MPO) plasma concentration, an enzyme secreted during inflammation by activated neutrophils,[17] has been found increased in patients with acute IS.[18] Inhibiting MPO activity led to a reduction in infarct size in animals.[19] Some data also suggest a detrimental effect of MPO activity on postischemic neurogenesis[20] and neuroprotection.[21] Furthermore, rtPA can promote in vitro and in vivo[16] release of early degranulation products from neutrophils, including MMP-9 and MPO.[22]

The aim of this study was to investigate, in patients with acute IS, the relationship between MMP-9 and MPO plasma concentrations and (1) baseline stroke severity, (2) circulating neutrophils and neutrophil-to-lymphocyte ratio (NLR), and (3) outcome at month 3. We also investigated MMP-9 and MPO release after ex vivo rtPA stimulation of neutrophils.

Methods

Inclusion and noninclusion criteria

We analyzed data collected in patients aged 40 years or more, with supratentorial IS, prospectively recruited within 48 hours of symptoms onset between June 2005 and May 2009 in the Biostroke study (NCT00763217), a study of biomarkers conducted in the stroke center of the Lille University Hospital. A subgroup of patients was recruited within 4.5 hours of symptoms onset in order to evaluate MMP-9 and MPO release after ex vivo rtPA treatment of neutrophils. Patients included in the Biostroke cohort who had either TIA or intracerebral hemorrhage were excluded from the analysis. Patients were not consecutive, because of the need to obtain written informed consent for all patients and the ability to perform the in vitro analysis only between 8 AM and 2 PM 3 days per week for the subgroup.

Clinical assessment

Stroke severity was assessed by the NIH Stroke Scale (NIHSS)[23] at inclusion in the study or just before the bolus of IV rtPA in patients who received thrombolytic therapy. Definitions used for variables included in the medical history, vascular risk factors, and evaluation of the baseline clinical characteristics were those used by our group in a previous study.[24] The outcome was assessed either at a face-to-face visit or by a telephone interview at 3 months using the modified Rankin Scale (mRS).[25]

IV thrombolysis administration

IV rtPA (Actilyse; Boehringer, Ingelheim, Germany) was administered according to the recommendations of the European Stroke Organisation[26] (i.e., 0.9 mg/kg body weight, maximum 90 mg, 10% of the dose as a bolus, followed by a 60-minute infusion).

Imaging

All patients in both cohorts underwent either a noncontrast CT scan (Somatom Sensation 16 detectors; Siemens, Erlangen, Germany) or an MRI scan (Philips Achieva [Philips, Best, the Netherlands], 1.5T, with fluid-attenuated inversion recovery, gradient echo T2*, diffusion b1000 with apparent diffusion coefficient mapping, and time-of-flight sequences) at admission. All patients underwent a second neuroimaging investigation by CT or MRI 6–10 days later in the Biostroke study and also 24–72 hours later in the subgroup, or at any time in case of clinical worsening. In patients who received IV rtPA, the second imaging investigation was performed between 22 and 36 hours after treatment.

Evaluation of neutrophil counts and total active MMP-9 and MPO plasma concentrations

Neutrophil and lymphocyte counts and total active MMP-9 and MPO plasma concentrations were determined on blood samples drawn at inclusion and after 72 hours in the subgroup. Blood samples from patients who received rtPA were collected before thrombolysis.

Plasma MMP-9 and MPO concentrations were determined as follows: all samples were collected into Vacutainer tubes (Becton Dickinson France; Le Pont de Claix, France). Plasma samples were obtained from tubes containing heparin and were kept frozen until analysis.

Total active MMP-9 levels (free proMMP-9 and free MMP-9 following activation using APMA) in the plasma were measured by the MMP-9 activity assay system (Fluorokine E, Human Active MMP-9; R&D Systems, Inc., Minneapolis,

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MN). Plasma MPO was measured by Magnetic Human High-Sensitivity Luminex assay (Millipore; St Quentin en Yvelines, France), according to the manufacturers' instructions. These assays were performed according to the recommendations of the manufacturer in duplicate the same day on the same run to allow direct comparisons.

End points

The 3 end points were (1) excellent outcome, defined as an mRS[25] score 0 or 1 at 3 months; (2) symptomatic intracerebral hemorrhage (sICH) according to the European Cooperative Acute Stroke Study–II definition[27]; and (3) death within 3 months.

Neutrophils isolation and ex vivo stimulation

Peripheral neutrophils degranulation was investigated at inclusion and before rtPA treatment for patients who received IV rtPA and after 72 hours for all patients. According to our previously described method,[28] neutrophils were isolated from patients' heparinized venous blood by centrifugation on Ficoll-Hypaque density gradient. Neutrophils resuspended in culture medium (Roswell Park Memorial Institute [RPMI] 1640 medium) were >98% pure, as determined by hematology analyzer (XS800i; Sysmex Corporation, Kobe, Japan). Then, purified neutrophils (4×10^5 cells per well) were seeded into 24-well plates and cultured in RPMI-1640 medium with L-glutamine and sodium pyruvate (Gibco BRL, Cheshire, UK) at 37°C, 5% CO_2. Neutrophils were incubated with 0.1 mg/mL rtPA for 30 minutes. Neutrophils supernatant was collected, centrifuged (12 minutes at 14,000 rpm), and stored at −80°C until analysis. Nontreated cells were used as controls. Total active MMP-9 (free proMMP-9 and free MMP-9) and MPO in conditioned media were measured as described above.

Statistics

Quantitative variables are expressed as mean (SD) in case of normal distribution or median (interquartile range) otherwise. Categorical variables are expressed as percentage (count). We compared groups for categorical variables, with the χ^2 test with Yates correction or Fisher exact test when appropriate, and for continuous variables with the Mann-Whitney *U* test. Normality of distributions was assessed using histograms and Shapiro-Wilk test.

We performed a backward multiple logistic regression analysis with excellent outcome, sICH, or death within 3 months as dependent variables. Independent variables were selected from the bivariate analysis for the excellent outcome, with a 0.10 level as a screening criterion for the selection of candidate variables. The variables age, NIHSS, neutrophil counts, MMP-9 and MPO plasma concentrations (model 1), and thrombolysis were forced into the model. MMP-9 and MPO plasma concentrations were entered in the model as categorical variables with 3 levels according to tertiles with initial reference category for a better interpretation of data. Concerning MPO plasma concentrations, cutoffs were set at 52.3 ×1,000 ng/mL for the 2nd tertile and at 91.8 ×1,000 ng/mL for the 3rd tertile, while for MMP-9 concentrations, cutoffs were set at 85 ng/mL for the 2nd tertile and at 159 ng/mL for the 3rd tertile. A variable computing interaction between MMP-9 and MPO tertiles was also entered (model 2). The same 2 analyses were repeated using NLR instead of neutrophil counts (model 3 and model 4, respectively). Subsequently other models were constructed with individual biomarkers instead of all combined: neutrophil counts (model 5) or NLR (model 6) or MMP-9 (model 7) or MPO (model 8) plasma concentrations, the latter 2 divided by tertiles. Correlations between variables were checked for possible colinearity, which was defined as r values >0.6. Data were analyzed using the SPSS 22.0 package for Windows and GraphPad Prism 6 for Windows.

Standard protocol approvals, registrations, and patient consents

The Biostroke study was approved by the local ethical committee. Patients were managed according to local rules without any investigation or treatment specifically performed. The study was explained fully to the patients. All patients or a close relative gave a signed informed consent for the dosages of MMP-9 and MPO. Neutrophil and lymphocyte counts were considered as part of usual care.

Data availability

All data used for analysis are presented in the tables and figure in this article. Data will be shared after ethics approval if requested by other investigators for purposes of replicating the results.

Results

Of 322 patients meeting inclusion criteria, 67 (20.8%) could not be analyzed because of missing data for delay of sample collection for neutrophils (5 patients) or plasma MMP-9 and MPO concentrations (20 patients), neutrophil count (19 patients), mRS at 3 months (32 patients), or any combination in the same patients. Finally, 255 patients were included in the analysis (median age, 70 [57–79] years; 116 women [45.1%]) and differed from patients excluded only for a lower proportion of women (45.5 vs 59.7%, $p = 0.038$), a lower baseline stroke severity (6 [2–13] vs 8 [2–19], $p = 0.026$), and no intrahospital death occurrence (0 vs 23.9%, $p < 0.001$).

Admission stroke severity and baseline characteristics of population

Of 255 patients, 137 (53.7%) had a low stroke severity (admission NIHSS score ≤5, table 1). Among demographic characteristics, stroke characteristics, and baseline clinical measures, only no history of congestive heart failure (9 ± 7.6 vs 22 ± 16.1, $p = 0.04$, for patients with admission NIHSS >5 and NIHSS ≤5, respectively), no rtPA treatment (41.6 vs 11.9%, $p < 0.001$), and lower admission glucose level (94.5 [86.7–109.7] vs 112 [95–131.5], $p < 0.001$) were associated with a lower stroke severity.

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Table 1 Baseline characteristics of the study population according to the initial stroke severity

	Baseline NIHSS score ≤5		
	No (n = 137)	Yes (n = 118)	*p* Value
Demographic characteristics			
Female sex	67 (48.9)	49 (41.5)	0.238
Age, y, median (IQR)	71 (58–79)	68 (55–79)	0.180
Body mass index, kg/m^2, median (IQR)	26.3 (23.3–29.4)	26.8 (22.9–29.4)	0.856
Medical history			
Arterial hypertension	82 (59.9)	78 (66.1)	0.304
Diabetes mellitus	31 (22.6)	20 (16.9)	0.258
Hypercholesterolemia	63 (46.0)	61 (51.7)	0.363
Coronary artery disease	32 (23.4)	21 (17.8)	0.275
Heart failure	22 (16.1)	9 (7.6)	0.040
History of atrial fibrillation	38 (27.7)	23 (19.5)	0.124
Recurrent stroke	19 (13.9)	9 (7.6)	0.112
Previous physical activity	65 (47.4)	68 (58.1)	0.090
Alcohol consumption	21 (15.4)	17 (14.4)	0.818
Smoking habit	35 (25.5)	38 (32.2)	0.241
Statins	48 (35.0)	32 (27.1)	0.174
Antithrombotic therapy	65 (47.4)	44 (37.3)	0.102
Stroke characteristics and treatment			
rtPA treatment	57 (41.6)	14 (11.9)	<0.001
Onset-to-treatment time, min, median (IQR)	140 (120–180)	130 (95–170)	0.400
Clinical and biological characteristics			
Systolic blood pressure, mm Hg[a]	150 (139.5–170)	155 (135–169)	0.871
Blood glucose concentration, g/L[a]	1.12 (0.95–1.31)	0.94 (0.87–1.10)	<0.001
Platelet count, ×1,000/mm^3, mean ± SD	244.1 ± 66.7	239.4 ± 76.8	0.604
Temperature, °C, mean ± SD	36.6 ± 0.6	36.7 ± 0.5	0.087
Delay of sample collection			
Onset-to-sample time, min, median (IQR)	1,260 (510–1,680)	1,500 (1,200–2,167)	<0.001
Outcome measures			
sICH	24 (17.6)	8 (6.8)	0.009
Death at day 8 (mRS 6)	0 (0.0)	0 (0.0)	—
Excellent outcome at 3 months (mRS 0–1)	35 (25.5)	95 (80.5)	<0.001
Death at 3 months (mRS 6)	23 (16.9)	2 (1.7)	<0.001
Biological measures			
Neutrophils, ×1,000/mm^3	6.4 (5.2–8.5)	4.6 (3.6–6.0)	<0.001
NLR	4.3 (2.8–6.6)	2.4 (1.8–3.6)	<0.001
MMP-9, ng/mL	125 (71–217)	116 (74.5–174)	0.195
MPO, ×1,000 ng/mL	77.9 (50.9–120)	57.5 (42–115)	<0.001

Abbreviations: IQR = interquartile range; MMP-9 = matrix metalloproteinase-9; MPO = myeloperoxidase; mRS = modified Rankin Scale; NIHSS = NIH Stroke Scale; NLR = neutrophil to lymphocyte ratio; rtPA = recombinant tissue plasminogen activator; sICH = symptomatic intracerebral hemorrhage (European Cooperative Acute Stroke Study–II definition).
Values are number (%) of patients, unless otherwise indicated.

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Admission stroke severity and biological parameters

Except for MMP-9 plasma concentrations, all biological parameters differed significantly between patients with and without admission NIHSS ≤5 (table 1). Patients with admission NIHSS ≤5 had lower neutrophil counts, NLR, and MPO plasma concentrations than patients with admission NIHSS >5. When the NIHSS was analyzed as a quantitative scale, NIHSS was positively correlated with neutrophils (r = 0.49, $p < 0.001$), NLR (r = 0.48, $p < 0.001$), and MPO plasma concentrations (r = 0.21, p = 0.001) but the correlation was not statistically significant for MMP-9 plasma concentrations (r = 0.06, p = 0.359).

rtPA and biological parameters

Patients treated by rtPA had higher neutrophil counts and higher NLR than patients not treated by IV rtPA (table 2). Conversely, MMP-9 and MPO plasma concentrations did not significantly differ between patients eligible or not for IV rtPA.

Adjusted and unadjusted correlation between neutrophil counts, NLR, and MPO and MMP-9 plasma concentrations

Neutrophil counts were significantly correlated with MPO as well as MMP-9 plasma concentrations in all patients (table 3). Moreover, the NLR was correlated with MPO plasma concentrations in all patients.

After adjustment for NIHSS and onset to sample delay, circulating neutrophil counts were significantly correlated with MMP-9 plasma concentrations in all patients (ρ = 0.215, p = 0.001). The NLR was significantly correlated with MMP-9 plasma concentrations (ρ = 0.146, p = 0.021).

Unadjusted correlation between functional outcome and biological measures

Except for MMP-9 plasma concentrations, all biological measures differed significantly between patients with and without excellent outcome (mRS 0–1) (table 4). Patients with excellent outcome had lower neutrophil counts, NLR, and MPO plasma concentrations than did patients with poor outcome. When the mRS was analyzed as a quantitative scale, mRS was positively correlated with neutrophils (r = 0.41, $p < 0.001$), NLR (r = 0.46, $p < 0.001$), and MPO (r = 0.30, $p < 0.001$) plasma concentrations, but the correlation was not statistically significant for MMP-9 plasma concentrations (r = −0.04, p = 0.518).

Influence of biological measures on outcome

Higher neutrophil counts were independently associated with a lower probability to have an excellent outcome, and a higher mortality rate at month 3, but were not associated with the risk of sICH. Higher tertiles for MPO plasma concentrations were associated with a lower probability to have an excellent outcome but were not associated with higher mortality rate and risk of sICH. MMP-9 plasma concentrations were not associated with any of these outcome measures (table 5). Indeed, the interaction of MMP-9 and MPO plasma concentrations, both divided by tertiles, was associated with a lower probability to have an excellent outcome only when the interaction of MMP-9 was with the highest tertile for MPO plasma concentrations (table 5). We did not observe the same behavior for highest tertiles of MMP-9 plasma concentrations. Only the interaction between intermediate tertile of MMP-9 and lowest tertile of MPO plasma concentrations was associated with lower probability of excellent outcome, but this finding was not stable since it was not replicable with other combinations of lower or intermediate tertiles (table 5).

These results remained similar in the 3 different models, with MMP-9 alone, MPO alone, and neutrophils alone. Results were not even modified by the replacement of neutrophil count by NLR alone or combined with MMP-9 and MPO plasma concentration or with their interaction (table 6).

Neutrophil isolation and degranulation

To better understand if MMP-9 and MPO could be considered as mediators of the neutrophil effects, we recruited 18 patients out of 255 (8 [44.4%] women, median age 80 years [74–88], and 12 patients [66.7%] received IV rtPA). Biology characteristics of this subgroup at baseline and after 72 hours are described in table 7. Patients who received IV rtPA were similar in terms of age and prestroke functional status as well

Table 2 Comparisons of biological cascade including neutrophils, neutrophil to lymphocyte ratio (NLR), matrix metalloproteinase-9 (MMP-9), and myeloperoxidase (MPO) plasma concentrations between patients eligible for IV recombinant tissue plasminogen activator (rtPA) or not

	rtPA treatment		
	Yes (n = 101)	No (n = 257)	*p* Value[a]
Neutrophils, ×1,000/mm^3	6.4 (4.9–8.5)	5.4 (3.9–6.9)	<0.001
NLR	4.2 (2.6–7.2)	3.0 (2.0–4.8)	0.001
MMP-9, ng/mL	140 (67–257)	117 (76–173)	0.246
MPO, ng/mL	69.6 (48.1–112.2)	65.2 (46.9–117.8)	0.520

Values are median (interquartile range).

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Table 3 Bivariate correlation between neutrophils or neutrophil to lymphocyte ratio (NLR) and matrix metalloproteinase-9 (MMP-9) or myeloperoxidase (MPO) plasma concentrations in all patients

	Neutrophils		NLR	
	ρ	*p* Value	ρ	*p* Value
All patients				
MMP-9	0.157	0.012	0.108	0.088
MPO	0.242	<0.001	0.217	0.001

as for stroke severity to patients who did not receive IV rtPA. HT was significantly more frequent in patients who received IV rtPA. In addition, MPO plasma concentrations were significantly higher at admission in patients eligible for IV rtPA compared to patients who were not eligible, while any difference was found at 72 hours between patients who received or not IV rtPA treatment. Any difference was found for neutrophil counts, NLR, and MMP-9 plasma concentrations at baseline and after 72 hours.

Neutrophil-derived MMP-9 and MPO concentrations

We therefore analyzed in this subgroup MMP-9 and MPO release after ex vivo rtPA stimulation of native neutrophils at admission and after 72 hours. At admission and before thrombolysis (H0), no differences were found in neutrophil-derived MMP-9 and MPO concentrations between patients treated or not by IV rtPA (figure, A and B). After 72 hours (H72), circulating neutrophils from patients treated by IV rtPA release more MMP-9 after rtPA stimulation than those not treated (568 ± 119 vs 229.8 ± 23.75, $p = 0.03$; figure, A). No differences were found for neutrophil-derived MPO at H0 and H72 between patients treated or not by IV rtPA (figure, B). Since there were no sICH cases in this subgroup, we considered this time any HT found at the control neuroimaging investigation. However, no significant differences were found between neutrophil-derived MMP-9 (figure, C) or MPO (figure, D) concentrations and HT according to treatment.

Table 4 Comparisons of biological measures including neutrophil counts, neutrophil to lymphocyte ratio (NLR), plasma matrix metalloproteinase-9 (MMP-9), and myeloperoxidase (MPO) concentrations between patients with and without excellent outcome (modified Rankin Scale [mRS] 0–1) at month 3

	mRS 0–1 at month 3		*p* Value
	Yes (n = 130)	No (n = 125)	
Neutrophils, ×1,000/mm^3	4.8 (3.7–6.1)	6.4 (5.2–8.5)	<0.001
NLR	2.6 (1.8–3.8)	4.5 (2.7–7.2)	<0.001
MMP-9, ng/mL	120 (75.7–188.2)	118 (69–194)	0.639
MPO, ×1,000 ng/mL	56.1 (41.9–88.9)	85.4 (53.9–136.5)	0.009

Values are median (interquartile range).

We explored the correlations between MMP-9 and MPO plasma concentrations, circulating neutrophil counts, NLR, and neutrophil-derived MMP-9 and MPO concentrations at corresponding time in whole population (table 8). Our results show that, at admission, neutrophil-derived MPO concentrations were negatively correlated with neutrophil-derived MMP-9. At H72, MPO plasma concentrations were positively correlated with NLR.

Discussion

Our study showed that, within the first 48 hours after symptoms onset in patients with supratentorial cerebral infarct, (1) circulating neutrophil counts, NLR, and plasma MPO concentrations were correlated with stroke severity but not total active MMP-9 plasma concentrations; (2) higher neutrophil counts were correlated with higher plasma MMP-9 concentrations in the whole cohort, even after adjustment for delay of sample collection and stroke severity; (3) higher neutrophil counts were correlated with plasma MPO concentrations in all patients; (4) neutrophil counts and NLR were higher in patients eligible for IV rtPA treatment but not total active MMP-9 plasma and MPO concentrations; (5) higher neutrophil counts and NLR were independently associated with poorer outcomes and higher mortality rate at 3 months, but not with sICH; (6) MPO plasma concentrations were associated with poorer outcomes, but not with sICH and mortality; and (7) total active MMP-9 plasma concentrations were not associated with any of the 3 end points.

To better understand if MMP-9 and MPO could be considered as mediators of the neutrophil effects,[5] we then focused our research on the neutrophil-derived MMP-9 and MPO. We found that (1) at H0, MPO plasma concentrations after ex vivo rtPA stimulation were higher in patients eligible for IV rtPA treatment but not associated with HT, but no differences were found concerning neutrophil-derived MPO concentrations in patients treated or not by IV rtPA; (2) after H72, circulating neutrophils from patients treated by IV rtPA release more MMP-9, but not MPO, after stimulation than those from patients not treated; (3) in the whole cohort, only neutrophil-derived MPO concentrations were found to be correlated negatively with neutrophil-derived MMP-9 at H0; and (4) MPO plasma concentrations were found to be correlated with NLR at H72.

Because the antigenic levels of MMP-9 or TIMP-1 alone may not always reflect the potential activity of MMP-9, total active

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Table 5 Results of the logistic regression analyses with excellent outcome (modified Rankin Scale score 0 or 1) at 3 months, death at month 3, and symptomatic intracerebral hemorrhage (sICH) as dependent variables in all patients

Dependent variables	Logistic regression analyses	Independent variables	adjOR (95% CI)	*p* Value	Variables entered in the model but not selected
Model 1 (with neutrophils + MMP-9 tertiles + MPO tertiles)					
Excellent outcome	Overall *p* value <0.001; well-classified 80.2%	Age; NIHSS ≤5; neutrophils[a]; hypertension; MPO T3/T1	0.96 (0.94–0.99); 15.26 (7.06–32.99); 0.36 (0.16–0.81); 0.36 (0.16–0.81); 0.37 (0.16–0.86)	0.005; <0.001; 0.031; 0.013; 0.021	MMP-9 all tertiles, MPO T2/T1, rtPA, female, CHF, AF, DM, SMK
sICH	Overall *p* value = 0.001; well-classified 87.3%	rtPA	4.02 (1.82–8.86)	0.001	Age, NIHSS ≤5, neutrophils, all MMP-9 and MPO tertiles
Death at month 3	Overall *p* value <0.001; well-classified 91.7%	Age; NIHSS ≤5; neutrophils[a]	1.09 (1.04–1.15); 0.15 (0.03–0.72); 8.60 (1.85–39.98)	<0.001; 0.017; 0.006	All MMP-9 and MPO tertiles, rtPA
Model 2 (with neutrophils + interaction between MMP-9 and MPO tertiles)					
Excellent outcome	Overall *p* value <0.001; well-classified 80.2%	Age; NIHSS ≤5; neutrophils[a]; hypertension; MPO T1*MMP-9 T2; MPO T3*MMP-9 T1; MPO T3*MMP-9 T2	0.96 (0.93–0.99); 18.48 (8.06–42.38); 0.29 (0.11–0.79); 0.11 (0.02–0.51); 0.38 (0.16–0.88); 0.06 (0.01–0.30); 0.17 (0.03–0.93)	0.004; <0.001; 0.015; 0.031; 0.005; 0.001; 0.041	Other combinations of MPO and MMP-9 tertiles, rtPA, female, CHF, AF, DM, SMK
sICH	Overall *p* value = 0.001; well-classified 87.7%	rtPA	3.94 (1.73–9.00)	0.001	Age, NIHSS ≤5, neutrophils, all 9 combinations of MPO and MMP-9 tertiles
Death at month 3	Overall *p* value <0.001; well-classified 93.7%	Age; NIHSS ≤5; neutrophils[a]	1.08 (1.02–1.14); 0.12 (0.02–0.58); 10.85 (1.89–62.45)	0.004; 0.009; 0.008	All 9 combinations of MPO and MMP-9 tertiles, rtPA

Abbreviations: adjOR = adjusted odds ratio; AF = atrial fibrillation; CHF = congestive heart failure; CI = confidence interval; DM = diabetes mellitus; MMP-9 = matrix metalloproteinase-9; MPO = myeloperoxidase; NIHSS = NIH Stroke Scale; rtPA = recombinant tissue plasminogen activator; SMK = smoking habit.
Model 1: with neutrophils, MMP-9, and MPO plasma concentrations divided by tertiles (T1 = 1st tertile, T2 = 2nd tertile, and T3 = 3rd tertile). Model 2: with neutrophils and interaction between MMP-9 and MPO plasma concentrations divided by tertiles.
[a] For 1,000 neutrophils/mm^3 increase.

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Table 6 Results of the logistic regression analyses with excellent outcome (modified Rankin Scale 0 or 1) at 3 months, death at month 3, and symptomatic intracerebral hemorrhage (sICH) as dependent variables in all patients

Dependent variables	Logistic regression analyses	Independent variables	adjOR (95% CI)	*p* Value	Variables entered in the model but not selected
Model 3 (with NLR + MMP-9 tertiles + MPO tertiles)					
Excellent outcome	Overall *p* value <0.001; well-classified 80.6%	Age; NIHSS ≤5; NLR; hypertension; MPO T3/T1	0.97 (0.94–0.97); 14.58 (6.69–31.78); 0.52 (0.30–0.93); 0.33 (0.15–0.75); 0.38 (0.16–0.88)	0.024; <0.001; 0.026; 0.008; 0.023	MMP-9 all tertiles, MPO T2/T1, rtPA, female, CHF, AF, DM, SMK
sICH	Overall *p* value = 0.001; well-classified 87.0%	rtPA	4.15 (1.88–9.17)	<0.001	Age, NIHSS ≤5, NLR, MMP-9 and MPO all tertiles
Death at month 3	Overall *p* value <0.001; well-classified 91.5%	Age; NIHSS ≤5; NLR	1.09 (1.04–1.15); 0.14 (0.03–0.67); 3.82 (1.49–9.78)	0.001; 0.014; 0.005	MMP-9 and MPO all tertiles, rtPA
Model 4 (with NLR + interaction between MMP-9 and MPO tertiles)					
Excellent outcome	Overall *p* value <0.001; well-classified 81.8%	Age; NIHSS ≤5; NLR; hypertension; MPO T1*MMP-9 T2; MPO T3*MMP-9 T1; MPO T3*MMP-9 T2; MPO T3*MMP-9 T3	0.97 (0.94–0.99); 17.90 (7.69–41.66); 0.45 (0.24–0.83); 0.37 (0.16–0.87); 0.09 (0.02–0.44); 0.06 (0.01–0.32); 0.15 (0.03–0.82); 0.19 (0.04–0.92)	0.016; <0.001; 0.010; 0.023; 0.003; 0.001; 0.028; 0.039	Other combinations of MPO and MMP-9 tertiles, rtPA, female, CHF, AF, DM, SMK
sICH	Overall *p* value = 0.001; well-classified 87.0%	rtPA	4.67 (2.06–10.59)	<0.001	Age, NIHSS ≤5, NLR, all combinations of MPO and MMP-9 tertiles
Death at month 3	Overall *p* value <0.001; well-classified 91.5%	Age; NIHSS ≤5; NLR	1.09 (1.04–1.15); 0.14 (0.03–0.67); 3.82 (1.49–9.78)	0.001; 0.014; 0.005	All combinations of MPO and MMP-9 tertiles, rtPA

Abbreviations: adjOR = adjusted odds ratio; AF = atrial fibrillation; CHF = congestive heart failure; CI = confidence interval; DM = diabetes mellitus; MMP-9 = metalloproteinase-9; MPO = myeloperoxidase; NIHSS = NIH Stroke Scale; NLR = neutrophil to lymphocyte ratio; rtPA = recombinant tissue plasminogen activator; SMK = smoking habit.
Model 3: with NLR, MMP-9, and MPO plasma concentrations divided by tertiles (T1 = 1st tertile, T2 = 2nd tertile, and T3 = 3rd tertile). Model 4: with NLR and interaction between MMP-9 and MPO plasma concentrations divided by tertiles. For the sake of brevity, data from models 5–8 are not shown in this table.

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Table 7 Biology characteristics at baseline and after 72 hours according to IV recombinant tissue plasminogen activator (rtPA) treatment

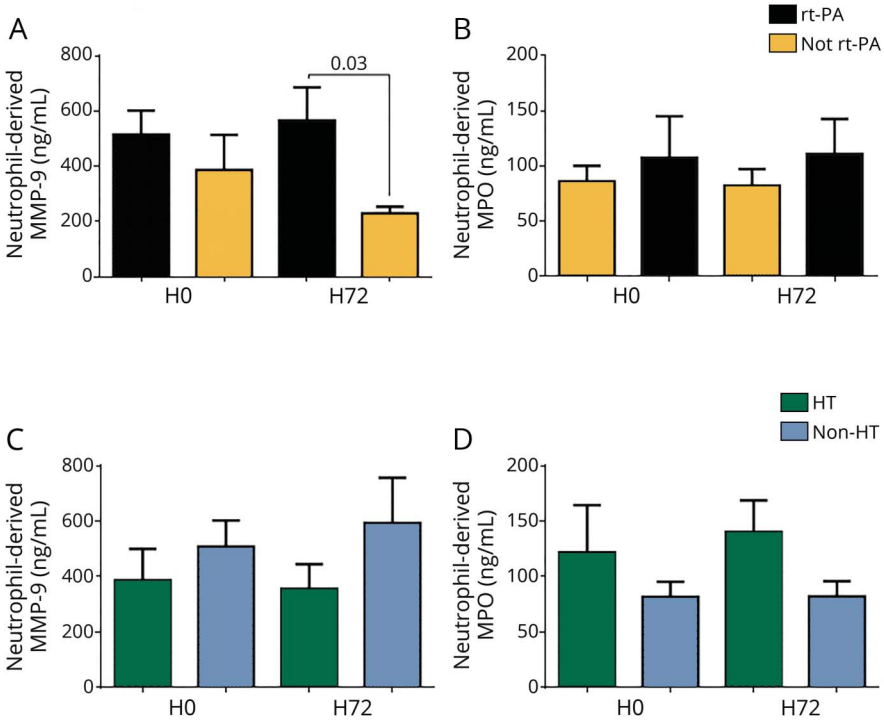
	H0			H72		
	rtPA (n = 12)	Not rtPA (n = 6)	*p* Value	rtPA (n = 12)	Not rtPA (n = 6)	*p* Value
Leukocytes, ×1,000/mm^3	9 (7.5–9.7)	7.6 (4.7–9.7)	0.2	8.8 (7–10.9)	6.7 (3.2–11.1)	0.2
Neutrophils, ×1,000/mm^3	6.8 (5.6–7.5)	5.6 (3.2–7.2)	0.2	6.5 (4.4–9.1)	3.2 (2.2–8.1)	0.1
NLR	6 (3–10)	5.3 (2.4–18.10)	0.6	5.9 (3.7–7)	3.2 (1.1–15.1)	0.2
Plasma MMP-9, ng/mL	121.4 (84.9–168.4)	135.8 (11.5–199)	0.5	130 (93–220)	103 (70.4–199)	0.6
Plasma MPO, ng/mL	69.9 (44.2–99)	32.9 (27.3–69.9)	0.03	77.2 (52–103)	62 (52.6–103)	0.5
NIHSS	11 (6–16)	7 (1–16)	0.15	6 (2–15.5)	3 (0.7–9.7)	0.3
HT				7 (58.3%)	1 (16%)	0.03

Abbreviations: HT = hemorrhagic transformation; MMP-9 = matrix metalloproteinase-9; MPO = myeloperoxidase; NIHSS = NIH Stroke Scale; NLR = neutrophil to lymphocyte ratio.

MMP-9 activity was measured. Our study was the first to evaluate the correlation between active form of plasma MMP-9 and neutrophil-derived MMP-9 levels and neutrophils in patients with acute cerebral ischemia within the first hours after symptoms onset.

The main limitation is the delay of several hours between symptoms onset and sample collection in the Biostroke study, with a possible selection bias due to the need for written informed consent. When analyses were adjusted on delays of sample collection concerning MMP-9, the results remained similar, but this may be insufficient to fully correct for the delay if the relationship is not perfectly linear. Thus, it is possible that peak of MMP-9 may have been missed in our study as the result of nonstatistical significance. Given the relatively brief lifespan of neutrophils in culture, the delay following their isolation from blood was a practical difficulty that limits the number of patients in the subgroup recruited

Figure Matrix metalloproteinase-9 (MMP-9) and myeloperoxidase (MPO) release from neutrophils according to recombinant tissue plasminogen activator (rtPA) treatment and hemorrhagic transformation (HT) occurrence



Histograms show the difference in MMP-9 and MPO release from neutrophils after ex vivo rtPA stimulation between patients treated by rtPA vs not treated (A, B) and in the HT group vs non-HT group (C, D).

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Table 8 Spearman (ρ) correlation between neutrophil-derived matrix metalloproteinase-9 (MMP-9) and myeloperoxidase (MPO) release and MMP-9 and MPO plasma concentrations, neutrophils, and neutrophil to lymphocyte ratio (NLR)

	Neutrophils	NLR	Plasma MMP-9	Neutrophil-derived MMP-9	Plasma MPO	Neutrophil-derived MPO
H0						
NLR	0.46					
Plasma MMP-9	−0.04	0.26				
Neutrophil-derived MMP-9	0.26	0.3	−0.24			
Plasma MPO	0.23	0.28	0.25	0.15		
Neutrophil-derived MPO	0.02	−0.17	0.03	−0.74[a]	−0.04	
HT	0.07	0.26	0.24	−0.24	0.05	0.31
H72						
NLR	0.64[a]					
Plasma MMP-9	0.16	0.23				
Neutrophil-derived MMP-9	0.43	0.03	0.25			
Plasma MPO	0.28	0.55[b]	0.43	0.14		
Neutrophil-derived MPO	0.42	0.19	−0.39	−0.14	−0.17	
HT	0.24	0.11	−0.31	−0.12	0.05	0.01

Abbreviation: HT = hemorrhagic transformation.
[a] Correlation is significant at the 0.01 level.
[b] Correlation is significant at the 0.05 level.

for studying the neutrophil degranulation. Another limitation is the lack of data on prestroke functional status, so that we could not perform a shift analysis between mRS prestroke and at 3 months.

In 2 previous studies, but not in ours, higher plasma MMP-9 concentrations were associated both in patients treated[13] and untreated by IV rtPA,[29] with worse outcomes,[29] especially sICH.[13] A possible explanation for this discrepancy could be the delay of sample collection, that is, less than 3[29] or 4.5 hours[13] vs up to 48 hours in our study: MMP-9 concentrations increase within the first 2–6 hours after symptom onset,[5,30] then remain stable over the first 48 hours after stroke,[7] as confirmed in our subgroup. However, it has also been reported that neither baseline MMP-9 concentrations measured within a few hours of IS onset nor the rate of MMP-9 at any point within 14 days had any association with the risk of HT in patients treated or not with lower dose of rtPA.[31] Another possible explanation could lie in the use of different definition of HT, that is, every HT[13] rather than sICH in our study.

Our study showed an influence of rtPA on neutrophil degranulation according to the kinetic and distribution pattern of the tertiary (MMP-9) and primary granules (MPO). This is consistent with evidence that rtPA plays a role on neutrophil degranulation and MMP-9 release, as shown in some in vitro experiments.[7,22]

No correlation was found between plasma MMP-9 concentrations and stroke severity in the whole Biostroke study cohort included within 48 hours. This finding could be connected to the short half-life (i.e., 1–2 hours) of rtPA, so that rtPA could influence the MMP-9 upregulation more in the first hours following its administration.[29] We cannot exclude that the sample size was too small for a subgroup analysis in patients treated by rtPA. Nevertheless, worse outcomes were statistically associated with higher neutrophil counts and NLR in the same sample, as previously described by us.[4]

The cellular source of MMP-9 is controversial.[32] If we consider the whole cohort, plasma MMP-9 concentrations were correlated with neutrophil counts and NLR. This finding together with the results of our in vitro experiments are consistent with the hypothesis that circulating neutrophils represent a major cellular source of circulating MMP-9.[33] Nevertheless, the lack of correlation between plasma MMP-9 concentration and the outcome in our study could be explained by the presence of other cellular sources in the acute

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phase of cerebral ischemia as well as resident brain cells, cells of the vasculature, or other circulating immune cells.[33] Another possible explanation for our result is the method used to measure MMP-9. Unlike other studies, the choice was made to measure the potential activity of total active MMP-9, the form responsible for tissue damage.

MPO plasma concentrations were associated with stroke severity, which agrees with earlier studies.[18,34] MPO plasma levels are elevated in patients with stroke compared with normal controls[18] and can predict future stroke and vasculopathic events in Fabry disease.[35] In our study, MPO plasma concentrations were correlated with neutrophil counts. MPO is the most abundant component in azurophilic (or primary) granules in neutrophils, which are the last to be released from the neutrophil by degranulation.[16] Indeed, in our study, the negative correlation between neutrophil-derived MMP-9 and MPO at admission could be due to the kinetics of activation of neutrophils in response to rtPA.[6] In fact, MPO activity peaks later, around 3–7 days after stroke.[17,19] Traditionally it has been used as a histopathologic marker for neutrophils. Indeed, it is now accepted that MPO is not an exclusive marker of neutrophils, but it is also expressed in the myeloid line, especially in monocytes and macrophages/microglia.[36]

Our study revealed a statistical association between neutrophil counts and NLR and MPO plasma concentrations, but not MMP-9, with stroke outcome. This could suggest that MMP-9 is probably not an interesting mediator of the deleterious effects of neutrophils on stroke severity and outcome. Higher MPO plasma concentrations are associated with worse functional outcome, but not with sICH and mortality. This observation is in line with a previous study that showed a role of MPO in predicting stroke severity.[18,37] Although our study showed an association but not a causative relationship between neutrophils and MPO, we could infer that MPO could be a potential therapeutic target in future studies.

Concerning post-thrombolysis hemorrhages, a selective inhibition of MMP-9[33,38] and MPO may be less promising than a global inhibition of neutrophils to improve outcomes in patients with cerebral ischemia.[39] Other potential mediators, such as elastase, reactive oxygen species, and other MMPs,[37] need to be evaluated.[5] The neutrophil is the key factor of post-thrombolysis hemorrhages.[4,39] Among all factors released by neutrophil, MMP-9 and MPO do not appear to be sufficient, although it remains to determine their place in addition or synergy with other released factors.[37,40]

Acknowledgment

The authors thank the Centre de Ressources Biologiques of Lille for technical assistance and contributions; and Valentina Panetta for statistical revision of the logistic regression model.

Study funding

This study was funded by the Programme Hospitalier de Recherche Clinique (Biostroke, Direction Générale de la Santé 2006/0153).

Disclosure

I. Maestrini, M. Tagzirt, S. Gautier, A. Dupont, A.M. Mendyk, S. Susen, A. Tailleux, E. Vallez, and B. Staels report no disclosures. C. Cordonnier reports receiving speaker fees from Boehringer-Ingelheim and Pfizer, is an investigator in clinical trials for Astra-Zeneca, Boehringer-Ingelheim, Daichi-Sankyo, and Servier, and is a member of advisory boards for BMS. D. Leys reports grants from Ministry of Health–Grant PHRC during the conduct of the study; receiving grant research from hospital and Adrinord in compensation of involvement in advisory boards and symposia from BMS/Pfizer, Boehringer-Ingelheim, and Bayer, outside the submitted work; and is Vice-editor of the *European Stroke Journal*. R. Bordet reports grants from Ministry of Health–Grant PHRC during the conduct of the study. Go to Neurology.org/N for full disclosures.

Publication history

Received by *Neurology* February 19, 2019. Accepted in final form December 10, 2019.

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Madjid Tagzirt, PhD	University of Lille, Inserm U1011, France	Collected data, analyzed and interpreted all data, performed the literature search, conceptualized the study, drafted the manuscript
Sophie Gautier, PharmD, PhD	University of Lille, Inserm U1171, France	Collected data, revised the manuscript
Annabelle Dupont, PharmD, PhD	University of Lille, Inserm U1011, France	Collected data, revised the manuscript
Anne-Marie Mendyk, RN	University of Lille, Inserm U1171, France	Collected data, conceptualized the study, revised the manuscript
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Anne Tailleux, PhD	University of Lille, Inserm U1011, France	Collected data, revised the manuscript
Emmanuelle Vallez	University of Lille, Inserm U1011, France	Collected data, revised the manuscript

Continued

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Appendix *(continued)*

Name	Location	Contribution
Bart Staels, PharmD, PhD	University of Lille, Inserm U1011, France	Conceptualized the study, revised the manuscript
Charlotte Cordonnier, MD, PhD	University of Lille, Inserm U1171, France	Conceptualized the study, revised the manuscript
Didier Leys, MD, PhD	University of Lille, Inserm U1171, France	Designed the study, analyzed and interpreted all data, drafted the manuscript, conceptualized the study
Regis Bordet, MD, PhD	University of Lille, Inserm U1171, France	Designed the study, interpreted all data, conceptualized the study, drafted and revised the manuscript

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Analysis of the association of MPO and MMP-9 with stroke severity and outcome: Cohort study

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Neurology 2020;95;e97-e108 Published Online before print February 28, 2020

DOI 10.1212/WNL.0000000000009179

This information is current as of February 28, 2020

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