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Circulating TIMP-1 is associated with hematoma volume in patients with spontaneous intracranial hemorrhage

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Matrix metalloproteinases (MMPs) are proteolytic zinc-endopeptidases regulated by tissue Inhibitors of matrix metalloproteinases (TIMPs). We evaluated the potential of MMPs and TIMPs as clinical tools for Intracranial Haemorrhage (ICH). Spontaneous non-traumatic ICH patients were recruited from two hospitals: Complejo Hospitalario de Navarra (CHN = 29) and Vall d' Hebron (VdH = 76). Plasmatic levels of MMP-1, -2, -7, -9, -10 and TIMP-1 and their relationship with clinical, radiological and functional variables were evaluated. We further studied the effect of TIMP-1 (0.05–0.2 mg/Kg) in an experimental tail-bleeding model. In CHN, TIMP-1 was associated with admission-hematoma volume and MMP-7 was elevated in patients with deep when compared to lobar hematoma. In VdH, admission-hematoma volume was associated with TIMP-1 and MMP-7. When data from both hospitals were combined, we observed that an increase in 1 ng/ml in TIMP-1 was associated with an increase of 0.14 ml in haemorrhage (combined $\beta = 0.14$, 95% CI = 0.08–0.21). Likewise, mice receiving TIMP-1 (0.2 mg/Kg) showed a shorter bleeding time ($p < 0.01$). Therefore, the association of TIMP-1 with hematoma volume in two independent ICH cohorts suggests its potential as ICH biomarker. Moreover, increased TIMP-1 might not be sufficient to counterbalance MMPs upregulation indicating that TIMP-1 administration might be a beneficial strategy for ICH.

Stroke is the second leading cause of death and disability worldwide¹. Intracranial hemorrhage (ICH), accounting for 10–15% of all strokes, is the most devastating subtype². However, unlike ischemic stroke, the incidence of ICH has not declined over the last decades³ and no specific treatments have been developed⁴. Almost 40% of ICH patients will die within the first 30 days and only 20% of survivors will achieve independent functionality⁵.

ICH is a complex disease characterized by blood vessel rupture and extravasation into brain parenchyma, leading to hematoma expansion, ischemia and neuronal loss⁶. The most common ICH are either lobar or deep in location and differ in admission hematoma volume, hematoma growth and outcome⁷. Several factors are involved in the ICH pathophysiology including: (a) the remodeling of the extracellular matrix (ECM) which in physiological conditions maintains the integrity, elasticity and strength of the arterial wall, (b) the breakdown of the blood-brain barrier (BBB) which contributes to edema formation and neuronal degeneration and (c) the inflammatory responses involved in secondary brain injury^{8,9}.

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	ICH	Lobar ICH	Deep ICH	p value
	(n = 29)	(n = 16)	(n = 13)	
Age (years)	76 [66–85]	72.5 [65–87]	82.0 [68–85]	0.442
Sex (Female), n (%)	11 (37.9)	7 (43.8)	4 (30.8)	0.474
Hypertension (Yes), n (%)	15 (51.7)	6 (37.5)	9 (69.2)	0.089
Diabetes (Yes), n (%)	7 (24.1)	3 (18.8)	4 (30.8)	0.452
Dyslipidemia (Yes), n (%)	11 (37.9)	7 (43.8)	4 (30.8)	0.474
Glucose (mg/mL)	133 [115–163]	133 [110–163]	128 [117–179]	0.834
Hematoma volume at admission (mL)	18 [7–46]	38 [19–69]	8 [4–17]	<0.001
Hematoma volume 24–48 h (mL)	20 [5–46]	42 [23–58]	9.4 [3.6–18]	0.004
Hematoma growth (Yes), n (%)	3 (11.5)	1 (7.7)	2 (15.4)	0.539
IVH (Yes), n (%)	9 (31)	7 (43.8)	2 (15.4)	0.101
mRS at admission (Score)	0 [0–0]	0.0 [0–1]	0.0 [0–0]	0.162
mRS 90 days (Score)	4 [2–6]	4.0 [3–6]	4.0 [2–6]	0.534
NIHSS at admission (Score)	8 [4–17]	9.0 [3–17.7]	5.0 [4–16.5]	0.965
Mortality 90 days (Yes), n (%)	9 (33.3)	6 (42.9)	3 (23.1)	0.276
MMP-1 (ng/mL)	6.3 [3.5–12.1]	4.8 [3.3–7.56]	9.7 [4.1–13.7]	0.200
MMP-2 (ng/mL)	110.5 [85.1–144.4]	105.6 [82–134.6]	111.6 [92.8–165]	0.382
MMP-7 (ng/mL)	17.3 [14–22.5]	15.3 [13–19]	21.1 [15.5–24.3]	0.048
MMP-9 (ng/mL)	237 [137.8–338]	257.3 [115.7–334.4]	223.8 [144–388]	1
MMP-10 (pg/mL)	577.9 [439.3–855.4]	485.1 [400–742.1]	589.6 [473.6–936.4]	0.216
TIMP-1 (ng/mL)	162.3 [139.8–225.1]	166.5 [122.1–232.1]	162.3 [146.5–222.4]	0.913

Table 1. Admission demographic and clinical characteristics of the complete CHN cohort (n = 29) after dividing by hematoma location (lobar n = 16, and deep n = 13). Continuous variables are presented as median [interquartile range]. Intraventricular Hemorrhage (IVH), modified Rankin Scale (mRS), National Institute of Health Stroke Scale (NIHSS). Bold numbers indicate statistically significant p-values.

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases with proteolytic activity that contribute to ECM degradation, tissue remodeling and regeneration¹⁰. The catalytic activity of MMPs is regulated by tissue inhibitors of matrix metalloproteinases (TIMPs)¹¹. The four members of the TIMP family (TIMP 1–4) are broad spectrum inhibitors that directly bind to the catalytic domain of MMPs¹².

After brain injury, the role of MMPs and their inhibitors may be critical, since both participate in early acute injury along with tissue recovering during later stages¹³. Clinical studies have observed increased MMP-2, -3, -9 and TIMP-1 levels in blood and cerebrospinal fluid of ICH patients when compared to normal population^{14,15}, suggesting that MMPs/TIMPs are likely to be involved in brain injury after spontaneous ICH and may be associated with patients' outcome¹⁶. In the last years our group has studied the role of MMPs in different neurovascular diseases. We showed that TIMP-1 levels were associated with mortality in patients with traumatic brain injury and malignant middle cerebral artery occlusion^{17–19}, while MMP-2 expression might be implicated in the development of amyloid angiopathy in ICH patients²⁰. Moreover, MMPs have been related to hematoma expansion, edema growth and neuronal loss in extensive pre-clinical studies¹⁶. However, their potential as therapeutic or diagnostic clinical tools has not yet been fully identified.

In this study, we explored the plasmatic levels of several MMPs and TIMP-1 and their relationship with hematoma volume and location as well as with other clinical, radiological and functional variables in two independent cohorts of ICH patients. In addition, we further studied the effect of TIMP-1 in an experimental tail-bleeding model.

Results

Clinical and demographic characteristics according to hematoma location. Clinical and demographic characteristics of both cohorts according to hematoma location at admission and follow-up data are shown in Tables 1 and 2.

In the CHN cohort (Table 1), the median [interquartile range, IQR] age was 76 [66–85] years, almost 40% were female, 52% had hypertension and the severity of the stroke was moderate (median [IQR], NIHSS = 8 [4–17]). Ninety days after ICH, the mortality rate reached 33% (n = 9) and survivors were moderate to severely disabled (mRS = 4 [2–6]). Patients with lobar ICH (n = 16, 55%) had larger hematoma volumes at admission when compared to deep location (38 [19–69] mL lobar vs. 8 [4–17] mL deep, $p < 0.001$), and 24–48 hours after ICH onset (42 [23–58] mL lobar vs. 9.4 [3.6–18] mL deep, $p = 0.004$). There was a tendency to a higher prevalence of hypertension (dichotomized) in patients with deep hematoma ($p = 0.089$). Only MMP-7 levels were higher in patients with deep hematoma when compared to lobar (median [IQR], 21.1 [15.5–24.3] ng/mL deep vs. 15.3 [13.0–19.0] ng/mL lobar, $p = 0.048$). We did not find differences on functional outcomes, mortality or the rest of MMPs and TIMP-1 between these two ICH locations (Table 1).

In the VdH cohort (Table 2), the median [IQR] age of ICH patients was 72 [66–80] years, nearly 44% were female, almost 90% presented hypertension and stroke severity ranged from moderate to severe (median [IQR], NIHSS = 12 [6.7–19.2]). Ninety days after ICH, mortality rate was 31% (n = 21) and survivors presented

	ICH	Lobar ICH	Deep ICH	p value
	(n = 76)	(n = 28)	(n = 48)	
Age (years)	72 [66–80]	77 [68–82]	69 [65–77]	0.060
Sex (Female), n (%)	33 (43.4)	12 (42.9)	21 (43.7)	1
Hypertension (Yes), n (%)	66 (86.8)	21 (75.0)	45 (93.7)	0.048
Diabetes (Yes), n (%)	21 (27.6)	10 (35.7)	11 (22.9)	0.348
Dyslipidemia (Yes), n (%)	46 (60.5)	19 (67.9)	27 (56.2)	0.450
Glucose (mg/mL)	128 [110–164]	141.5 [117–216.5]	124 [108–148]	0.037
Hematoma volume at admission (mL)	10.6 [3.9–35]	43.7 [15.5–77]	5.8 [3.2–19.3]	<0.0001
Hematoma volume 24–48 h (mL)	8 [3.6–37.1]	39 [20.2–48.1]	5.7 [3.3–14.8]	0.001
Hematoma growth (Yes), n (%)	12 (27.3)	3 (20.0)	9 (31.0)	0.673
IVH (Yes), n (%)	32 (42.1)	12 (42.9)	20 (41.7)	1
mRS at admission (Score)	0 [0–2]	0 [0–2]	0 [0–1.7]	0.782
mRS 90 days (Score)	4 [2–6]	5 [2–6]	3 [2–5.7]	0.198
NIHSS at admission (Score)	12 [6.7–19.2]	15 [9–21]	11 [6–19]	0.227
Mortality 90 days (Yes), n (%)	21 (31.3)	11 (45.8)	10 (23.3)	0.102
MMP-1 (ng/mL)	7.245 [3.9–11.9]	6.09 [2.9–11.5]	8.33 [5.4–12.3]	0.171
MMP-2 (ng/mL)	106.4 [83.7–116.6]	108.7 [83.7–115.3]	105.17 [83.7–117.0]	1
MMP-7 (ng/mL)	20.7 [16.0–27.6]	23.9 [16.3–31.8]	19.5 [16.0–26.4]	0.208
MMP-9 (ng/mL)	110.1 [78.3–160.4]	121.6 [78.3–205.1]	106.4 [77.6–140.4]	0.306
MMP-10 (pg/mL)	478.9 [297.7–710.4]	492.0 [290.4–963.0]	478.9 [297.7–599.2]	0.410
TIMP-1 (ng/mL)	202.9 [169.7–243.5]	225.4 [174.1–249.7]	199.2 [169.3–235.3]	0.294

Table 2. Admission demographic and clinical characteristics of the complete VdH cohort (n = 76) after dividing by hematoma location (lobar n = 28, and deep n = 48). Continuous variables are presented as median [interquartile range]. Intraventricular Hemorrhage (IVH), modified Rankin Scale (mRS), National Institute of Health Stroke Scale (NIHSS). Bold numbers indicate statistically significant p-values.

moderate to severe disability (mRS = 4 [2–6]). ICH location was lobar in 28 patients (37%) and deep in 48 (63%). As reported for the CHN cohort, hemorrhage volumes were also larger in patients with lobar hematoma when compared to deep location at admission (43.7 [15.55–77] mL lobar vs. 5.8 [3.25–19.27] mL deep, $p = 0.0001$) and 24–48 hours after ICH (39 [20.2–48.11] mL lobar vs. 5.7 [3.3–14.8] mL deep, $p = 0.0015$). Furthermore, the prevalence of hypertension was higher in patients with deep hematoma when compared to lobar (94% deep vs. 75% lobar, $p = 0.047$), while glucose levels were lower (124 [108–147.75] deep vs. 141.5 [116.75–216.5] mg/mL lobar, $p = 0.036$). No significant association of hematoma location was observed with any of the functional outcomes, mortality, MMPs or TIMP-1 (Table 2).

Association of MMPs and TIMP-1 with clinical and radiological variables. We performed a univariate analysis to assess the relationship between plasma MMPs and TIMP-1 and clinical and radiological variables (Tables 3 and 4).

First in the CHN cohort, the MMPs studied were not associated with either cardiovascular risk factors, functional outcomes or mortality (Supplemental Table 1). However, TIMP-1 levels were positively associated with admission hematoma volume (0.19; 95% CI 0.09 to 0.28 $p = 0.001$, Table 3). We also assessed the association between admission hematoma volume and different clinical and radiological parameters. Admission NIHSS (2.8; 95% CI 1.1 to 4.4, $p = 0.002$), 90-days mortality (46; 95% CI 12.5 to 79.5, $p = 0.009$) and ventricle's blood (44.2; 95% CI 12.2 to 76.2, $p = 0.008$) were positively associated with admission hematoma volume. Moreover, patients with a lobar location had a significantly higher hemorrhage volume than patients with a deep location (43.7; 95% CI 14.5 to 72.8, $p = 0.005$). Hypertensive patients showed lower hematoma volume (−32.2; 95% CI −63.4 to −0.94, $p = 0.044$, Table 3).

In the VdH cohort, MMP-2 levels were associated with age (0.95; 95% CI 0.27 to 1.63, $p = 0.007$), mRS 90 days (3.8; 95% CI 0.48 to 7.07, $p = 0.025$), hypertension and 90-days mortality (OR = 1.032, 95% CI 1.002 to 1.064, $p = 0.039$ and OR = 1.025, 95% CI 1.003 to 1.048, $p = 0.027$, respectively). MMP-10 levels were associated with age (11.7; 95% CI 1.4 to 21.9, $p = 0.025$) and diabetes (OR = 1.001, 95% CI 1.000 to 1.003, $p = 0.021$), and MMP-9 levels were lower in female patients (−59.9; 95% CI −98.1 to −7.7, $p = 0.022$, Supplemental Table 2). Moreover, we again observed that admission hematoma volume was positively associated with TIMP-1 levels (0.17; 95% CI 0.03 to 0.32, $p = 0.022$, Table 4) but also with MMP-7 levels (0.83; 95% CI 0.32 to 1.34, $p = 0.002$, Table 4). Regarding the association between admission hematoma volume and other clinical and radiological parameters, we found a positive association of this variable, with glucose levels (0.25; 95% CI 0.1 to 0.41 $p = 0.0021$), age (1.3; 95% CI 0.4 to 2.3, $p = 0.007$), mortality (48.7; 95% CI 29.4 to 68.0, $p < 0.001$) and with admission NIHSS (2.05; 95% CI 0.99 to 3.11, $p < 0.001$). Again, patients, with lobar location had a significantly higher hemorrhage volume than patients with a deep location (46.2; 95% CI 29.9 to 62.6, $p < 0.001$). Hypertensive patients showed lower hematoma volume (−28.94; 95% CI −55.1 to −2.78, $p = 0.033$, Table 4).

	B (95% CI)	Beta	p value
Age (years)	0.52 (−0.87–1.90)	0.15	0.450
Sex (Female)	−6.1 (−40.7–28.5)	−0.07	0.721
Hypertension	−32.2 (−63.4–0.94)	−0.38	0.044
Diabetes	−28.1 (−65.9–9.7)	−0.28	0.139
Dyslipidemia	−22.9 (−56.4–10.7)	−0.26	0.173
Glucose (mg/mL)	0.22 (−0.17–0.60)	0.22	0.262
Hematoma location (Lobar)	43.7 (14.5–72.8)	0.51	0.005
Hematoma growth	−9.1 (−32.4–14.2)	−0.16	0.426
IVH	44.2 (12.2–76.2)	0.48	0.008
mRS at admission (Score)	−11.9 (−40.6–16.6)	−0.16	0.398
mRS 90 days (Score)	8.0 (−0.23–16.2)	0.37	0.056
NIHSS at admission (Score)	2.8 (1.14–4.41)	0.56	0.002
Mortality 90 days	46.0 (12.5–79.5)	0.49	0.009
MMP-1 (ng/mL)	0.31 (−1.06–1.70)	0.10	0.639
MMP-2 (ng/mL)	0.15 (−0.23–0.53)	0.16	0.419
MMP-7 (ng/mL)	−1.69 (−3.80–0.42)	−0.32	0.112
MMP-9 (ng/mL)	−0.01 (−0.14–0.13)	−0.02	0.934
MMP-10 (pg/mL)	0.02 (−0.03–0.07)	0.14	0.480
TIMP-1 (ng/mL)	0.19 (0.09–0.28)	0.60	0.001

Table 3. Association of admission hematoma volume with clinical parameters and MMPs and TIMP-1 levels in the CHN cohort (n = 29). Intraventricular Hemorrhage (IVH), modified Rankin Scale (mRS), National Institute of Health Stroke Scale (NIHSS). Bold numbers indicate statistically significant p-values.

TIMP-1 as a potential biomarker for hematoma volume.

We aimed to further analyze the association of plasmatic TIMP-1 with the volume of hemorrhage at admission observed in both cohorts by multivariate regression analysis.

First, in the CHN cohort, the multivariate analysis showed a significant association of TIMP-1 and hemorrhage volume after adjusting for confounding factors in all tested models (models 1 to 3, Table 5). This association was further confirmed in the VdH cohort in both, the univariate and the multivariate analysis (Table 6).

When we combined the results from the two cohorts, we observed a statistically significant association between TIMP-1 and hematoma volume (Overall $\beta = 0.14$, 95% CI = 0.08–0.21, Fig. 1). These results suggest that TIMP-1 might be a potential biomarker for ICH.

TIMP-1 effects in experimental haemorrhage.

After finding an association between hematoma volume and TIMP-1, we further explored the effects of TIMP-1 in an experimental model of tPA-induced haemorrhage. Our results showed that an experimental dose of TIMP-1 (0.05 mg/Kg) did not decrease the maximum bleeding time allowed (30 minutes). However, mice injected with the highest dose of TIMP-1 (0.2 mg/Kg), showed a much shorter bleeding time (1.8 ± 0.7 vs. 28.3 ± 3.3 min, $p < 0.01$, Fig. 2a) and blood lost (15.3 ± 4.7 vs. $42.8 \pm 14.9 \mu\text{L}$, $p < 0.05$, Fig. 2b). These results suggest that TIMP-1 might be able to control experimental haemorrhage.

Discussion

The main objective of this study was to explore the relationship of MMPs and TIMP-1 with clinical, radiological and functional variables in two independent cohorts of ICH patients. Hematoma volume and expansion have been associated with poor prognosis, neurological detriment and disability after ICH²¹. We reported for the first time that circulating levels of TIMP-1 at admission are associated with hematoma volume independently of hypertension, NIHSS and hematoma location in two independent cohorts of ICH patients.

TIMP-1 is an endogenous inhibitor of MMPs present under normal conditions in tissues to regulate MMPs activity²². Studies addressing the role of TIMP1 in ICH are scarce. In this study, we report that TIMP-1 is significantly associated with hematoma volume in two independent cohorts, and after a combined meta-analysis, suggesting that TIMP-1 might be potential critical biomarker in ICH. These results are in agreement with other studies showing increased concentrations of TIMP-1 during the acute phase of ICH, that remained highly sustained during the first week after stroke onset²³, and showing TIMP-1 association with early mortality rates²⁴, supporting a potential role of TIMP-1 as severity biomarker in ICH. The elevated secretion of TIMP-1 might be induced to lessen MMPs activity and thus, to prevent further brain damage²⁵. However, it might not be enough to counterbalance the concomitant upregulation of MMPs. Likewise, it has been reported a relative increase in MMP-9/TIMP-1 ratio associated with symptomatic ICH²⁶ and Subarachnoid Haemorrhage (SAH)²⁷ supporting the hypothesis that perturbations in MMPs/TIMPs levels may contribute to the matrix disruption associated with cerebral aneurysms²⁸.

Additionally, we found that administration of TIMP-1 (approximately 10-fold circulating levels) is able to control experimental acute bleeding in mice. These findings are consistent with the overall idea that in conditions of brain haemorrhage, damage, and inflammation, there may be an imbalance in the dynamic equilibrium between MMPs and TIMPs, so that any way of enhancing the inhibitor side of the equation might be beneficial^{29,30}.

	B (95% CI)	Beta	p value
Age (years)	1.33 (0.4–2.27)	0.32	0.007
Sex (Female)	−3.24 (−22.02–15.53)	−0.04	0.736
Hypertension	−28.94 (−55.1–2.78)	−0.25	0.033
Diabetes	5.12 (−15.36–25.61)	0.06	0.625
Dyslipidemia	−14.28 (−32.97–4.41)	−0.18	0.139
Glucose (mg/mL)	0.25 (0.1–0.41)	0.35	0.002
Hematoma location (Lobar)	46.22 (29.87–62.58)	0.55	<0.001
Hematoma growth	−12.87 (−26.32–0.58)	−0.28	0.068
IVH	17.7 (−0.64–36.03)	0.22	0.063
mRS at admission (Score)	6.11 (−0.9–13.12)	0.20	0.092
mRS 90 days (Score)	10.9 (6.01–15.79)	0.50	<0.001
NIHSS at admission (Score)	2.05 (0.99–3.11)	0.41	<0.001
Mortality 90 days	48.72 (29.43–68.02)	0.53	<0.001
MMP-1 (ng/mL)	0.45 (−0.96–1.86)	0.07	0.534
MMP-2 (ng/mL)	0.15 (−0.15–0.46)	0.12	0.324
MMP-7 (ng/mL)	0.83 (0.32–1.34)	0.36	0.002
MMP-9 (ng/mL)	0.05 (−0.04–0.14)	0.12	0.308
MMP-10 (pg/mL)	0.01 (−0.01–0.03)	0.14	0.236
TIMP-1 (ng/mL)	0.17 (0.03–0.32)	0.27	0.022

Table 4. Association of admission hematoma volume with clinical parameters and MMPs and TIMP-1 levels in the VdH cohort (n = 76). Intraventricular Hemorrhage (IVH) modified Rankin Scale (mRS), National Institute of Health Stroke Scale (NIHSS). Bold numbers indicate statistically significant p-values.

	Model 1 (TIMP-1, hematoma location and HTA)		Model 2 (TIMP-1, hematoma location and NIHSS)		Model 3 (TIMP-1, hematoma location, HTA and NIHSS)	
	B (95% CI)	p value	B (95% CI)	p value	B (95% CI)	p value
TIMP-1	0.16 (0.08–0.25)	0.001	0.13 (0.04–0.21)	0.004	0.13 (0.04–0.21)	0.004
Location (lobar)	30.37 (5.9–54.9)	0.017	34.48 (13.3–55.7)	0.003	32.8 (10.1–55.6)	0.007
HTA	−19.38 (−43.5–4.7)	0.111			−5.8 (−31.1–19.5)	0.641
NIHSS			1.85 (0.56–3.15)	0.007	1.7 (0.19–3.19)	0.029

Table 5. Multivariate linear regression analysis for admission hematoma volume (dependent variable) in the CHN cohort. Independent variables, model 1 includes: TIMP-1, hematoma location, and hypertension (HTA); model 2: TIMP-1, hematoma location and NIHSS; and model 3: TIMP-1, hematoma location, hypertension (HTA) and NIHSS.

Supporting this hypothesis, previous data in TIMP-1 knockout mice showed exacerbated BBB disruption and brain injury³¹, while adenoviral TIMP-1 overexpression in mice, reduced brain lesion volume, neuronal damage and BBB leakage after ischemia³². Moreover, MMP inhibitors significantly decreased bleeding and brain damage in experimental models of haemorrhage, including ICH^{33,34}.

One of the main factors that influences ICH outcome is the hematoma location; deep and lobar predominantly³⁵ that usually results in different hematoma volumes. Despite the relevance of this parameter for ICH outcome, there are few clinical data regarding the association of MMPs with hematoma location. Abilleira *et al.* reported higher MMP-9 plasmatic levels in patients with deep-associated ICH³⁶. Moreover, in cerebral amyloid ICH, typically located in lobar regions, MMP-2 and -9 were up-regulated in perihematoma area of the brains^{20,37}. In our study, CHN patients with deep hematoma location showed elevated plasmatic MMP-7 levels compared to patients with lobar hematoma. MMP-7/matrilysin is a small, secreted metalloproteinase capable of digesting a large series of proteins of the extracellular matrix³⁸. A recent population-based cohort study followed for more than 10 years, reported that high serum levels of MMP-7 were independently associated with increased risk of incident spontaneous SAH suggesting its possible use as a predictive marker for SAH³⁹. Likewise, several studies with MMP-7 null mice indicated that the robust expression of MMP-7 during severe inflammation lead to irreparable tissue damage⁴⁰. Furthermore, we observed an association of this metalloprotease with hematoma volume in the VdH cohort. Therefore, our results suggest that MMP-7 might be involved in the pathophysiology of deep-ICH although it may be partly dependent on risk factors, therefore, further studies must be performed to confirm these data.

Other associations found in this study, such as MMP-10 with age and diabetes and MMP-2 with age, hypertension and 90-days mortality, were only significant in VdH possibly explained by larger sample size and statistical power of this cohort. However, these associations were lost after adjusting for risk factors of ICH. Previous data about the association of high MMP-10 levels with microvascular complications in diabetes type 1 in clinical and

	Model 1 (TIMP-1, hematoma location and HTA)		Model 2 (TIMP-1, hematoma location and NIHSS)		Model 3 (TIMP-1, hematoma location, HTA and NIHSS)	
	B (95% CI)	p value	B (95% CI)	p value	B (95% CI)	p value
TIMP-1	0.15 (0.03–0.27)	0.018	0.17 (0.06–0.28)	0.003	0.17 (0.06–0.28)	0.003
Location (lobar)	42.03 (25.36–58.69)	<0.001	42.15 (28.18–56.13)	<0.001	41.24 (26.52–55.96)	<0.001
HTA	–12.58 (–35.53–10.38)	0.287			–4.34 (–24.92–16.24)	0.681
NIHSS			1.99 (1.16–2.82)	<0.001	1.96 (1.11–2.81)	<0.001

Table 6. Multivariate linear regression analysis for admission hematoma volume (dependent variable) in the VdH cohort. Independent variables, model 1 includes: TIMP-1, hematoma location, and hypertension (HTA); model 2: TIMP-1, hematoma location, NIHSS; and model 3: TIMP-1, hematoma location, hypertension (HTA), and NIHSS.

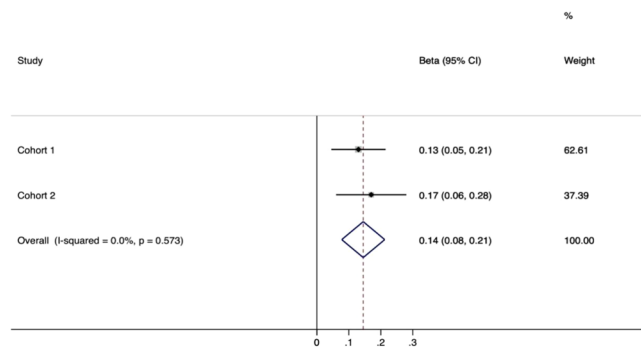


Figure 1. Fixed effect meta-analysis to assess the effect of admission TIMP-1 and hematoma volume in ICH patients. Beta coefficients were adjusted for location, hypertension and NIHSS in both cohorts (Cohort 1 = CHN and Cohort 2 = VdH).

experimental studies^{41,42} could explain the association found in ICH patients. Regarding MMP-2, several studies support the association of MMP-2 with hypertension and mortality in diabetic patients and in patients with heart failure^{43,44}. However, concerning spontaneous ICH patients, elevated levels of MMP-2 were only found at admission decreasing during the first 24 hours of hospitalization²³ and, specifically at tissue level in β -amyloid-damaged vessels located far from the acute ICH²⁰. Therefore, future studies with larger sample size might confirm these data.

Our work has several limitations: first the small sample sizes of our series make our results to be interpreted carefully. Therefore, data from this study should be confirmed in larger cohorts before definitive clinical conclusions can be drawn. The observational study design did not allow us to establish a cause-effect relationship between TIMP-1 and hematoma volume, although the effect of TIMP-1 reducing bleeding time in an experimental model of tail bleeding supports the hypothesis that TIMP-1 could be a promising strategy to control hematoma volume in ICH. However, further experimental models of ICH using deficient mice might be performed.

Conclusion

We report for the first time that circulating TIMP-1 is associated with hematoma volume in two independent ICH cohorts and when we combined the results from both cohorts suggesting the potential of TIMP-1 as biomarker for ICH. Additionally, TIMP-1 administration significantly reduces experimental bleeding. Therefore, it could be considered that after ICH increased TIMP-1 levels might not be sufficient to counterbalance the upregulation of MMPs, and that its exogenous administration could be a beneficial strategy for ICH in order to reduce MMPs activity and restore the MMPs/TIMPs balance.

Materials and methods

Study populations. Spontaneous non-traumatic ICH patients were recruited from two different Spanish tertiary hospitals (Tables 1 and 2): a cohort from Complejo Hospitalario de Navarra (CHN, n = 29), collected between February 2016 and June 2017; and a larger cohort of patients (n = 76) from Hospital Universitario Vall d'Hebron (VdH) collected between August 2012 and December 2013. These prospective studies were approved by the ethics committee of the Navarra Government (63/2015) and the Catalonia Government [PR(AG)157–2011] respectively. All patients or their “legally authorised representative” provided written informed consent according to Helsinki declaration. The study included only patients with spontaneous nontraumatic supratentorial intracerebral hemorrhage (ICH), diagnosed by experienced neurologists of the Stroke Unit by initial cranial non-contrast computed tomography (CT) at admission in the emergency department. Patients with brainstem/infratentorial ICH and/or secondary ICH (vascular malformation, aneurysm, tumors, ischemic stroke with haemorrhagic transformation) were excluded. We excluded other causes of intracranial haemorrhages, as subdural hematoma, epidural bleeds, and subarachnoid hemorrhage. However, we included patients who had suffered subarachnoid and intraventricular haemorrhage (IVH), although always associated with concurrent parenchymal hematoma.

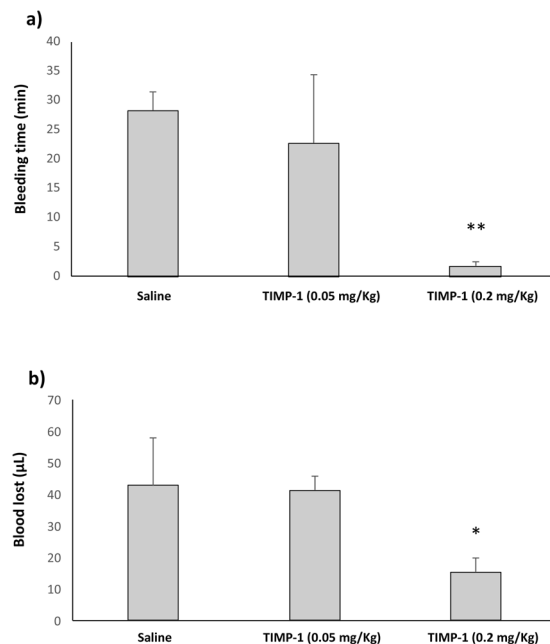


Figure 2. TIMP-1 effects in mice experimental tail bleeding. (a) Bleeding time of animals treated with saline (n = 5), TIMP-1 (0.05 mg/Kg, n = 4) and TIMP-1 (0.2 mg/Kg, n = 3). (b) Blood lost obtained after experimental bleeding model. **p < 0.01 and *p < 0.05 vs. Saline.

Systemic cancer and inflammatory systemic condition patients were also excluded from the study. ICH was classified by its location by experienced neurologist as previously reported by Aguilar I *et al.*⁴⁵. Demographics and previous antiplatelet and/or anticoagulation therapy were registered. Reversal of anticoagulation was indicated in some patients according to current guidelines.

Radiologic measurements. The primary radiologic outcomes were hemorrhage volume (admission volume and after the following 24–48 hours), hematoma location, hematoma expansion (more than 6 mL or 33% growth during the first 24 hours) and IVH. Hematoma volume was quantified by using the ABC/2 method^{46,47}. This well-validated method, consist on measuring the greatest axial dimension of the hematoma (A), a perpendicular diameter on the same slice (B), and the cranio-caudal dimension (C) estimated by counting the number of slices where the hematoma is present and multiplying that number by the slice thickness. The hematoma volume is estimated by multiplying these three dimensions together and dividing by 2, which estimates the simplified volume of an ellipse.

Neurological and functional outcomes. Clinical severity was assessed at admission using the National Institute of Health Stroke Scale (NIHSS)⁴⁸ and initial prognosis was evaluated by ICH score⁴⁹. Functional outcome was defined by modified Rankin Scale (mRS)⁵⁰ and mortality rates at 90 days after onset to evaluate the mid-term outcome of patients. Previous functional situation evaluated by mRS was registered at admission.

Sample collection. Citrated plasma samples of venous blood were obtained from both study cohorts at the moment of hospitalization (admission). All samples were processed within 4 hours of collection and stored at -80°C . Samples were thawed on ice and thoroughly vortexed prior to beginning any experiment. Plasma MMP-1, -2, -7, -9, -10 were measured with MAP Human MMP panel 2 (Milliplex, Millipore, Darmstadt, Germany) in a Luminex 200 platform (Luminex technology, Comercial Rafer S.L., Spain) and TIMP-1 was measured with ELISA kit for TIMP-1 (R&D Systems, Minneapolis, US) in an automated ELISA system (Triturus, Movaco, Spain). All experiments were performed according to manufacturer's instructions by blinded investigators.

Bleeding assay. Bleeding assay was performed in 5-week-old male C57/BL6J mice (n = 12, Envigo, Spain). All experiments in this study were conducted according to the European Community guidelines for the ethical animal care and use of laboratory animals (2010/63/EU) and approved by the University of Navarra Animal Research Review Committee (ref. ^{46,12}). The experimental protocol is shown in Supplemental Fig. 1. Briefly, animals were anesthetized with 2.5% isoflurane, placed in prone position and tPA (0.05 mg/Kg) was injected through the ocular plexus. Five minutes later, TIMP-1 (0.05 and 0.2 mg/Kg) or saline were injected through the femoral vein catheter (10% bolus and 90% perfusion during 40 minutes). A distal 5 mm segment of the tail was transected with a scalpel blade 5 minutes after starting the treatments. The tail was immediately immersed in a tube containing pre-warmed saline (37°C in a water bath). Bleeding time was defined as the time elapsed until bleeding stops for a maximum of 30 min. Blood lost was defined as the amount of blood collected during 30 min and absorbance

was measured in a spectrophotometer (Tecan Sunrise, Switzerland) and quantified against a standard curve generated with known quantities of blood³³.

Statistical analysis. Descriptive statistics (median, interquartile range, frequency and percentage) were defined for all clinical, radiological and functional variables. Normality was assessed using the Shapiro-Wilk test. Bivariate comparisons of characteristics at admission between ICH location (deep and lobar index ICH) were performed using Student t test or Mann-Whitney U test for continuous variables, and χ^2 test or Fisher exact test for categorical variables. All covariates associated with the end point in the univariate analysis, considering a threshold of $p < 0.1$ in both cohorts, were included in the multivariate linear regression analyses for the target molecules of the study (MMPs and TIMP-1). Due to the small sample size in one of the cohorts, the following baseline models were considered for adjustment: model 1, TIMP-1, location, and hypertension (yes/no); and model 2, TIMP-1, location and NIHSS. Finally, we conducted a fixed effect meta-analysis to combine the data from our two cohorts in a meta-analysis. Statistical analysis of the data from experimental model was done using Mann-Whitney U two-tailed test.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

M.N.O. participated in the design of the project, experimental work, statistical analysis, and wrote, reviewed and edited the manuscript; R.M. participated in the design of the project and edited and reviewed the manuscript; B.Z. participated in the design of the project and reviewed the manuscript; J.M.E. participated in the design of the project and reviewed the manuscript; A.B.P. participated in the design of the project and reviewed the manuscript; J.A.R. participated in the design of the project and reviewed the manuscript; C.R. participated in the design of the project and reviewed the manuscript; J.A.P. participated in the design of the project, supervised the work and edited and reviewed the manuscript; E.T. participated in the statistical analysis and reviewed the manuscript; J.M. participated in the design of the project and reviewed the manuscript; M.H.G. participated in the design of the project and edited and reviewed the manuscript; J.O. was in charge of the whole project design, supervised the work and wrote, edited and reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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