Sex-specific Association of Matrix Metalloproteinases with Secondary Injury and Outcomes after Intracerebral Hemorrhage

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Objective: Intracerebral hemorrhage affects approximately 2 million individuals per year. While the incidence is roughly equal in men and women, few studies have examined the influence of sex on secondary injury and associated long-term functional outcomes. Matrix metalloproteinases (MMPs) promote vessel rupture and worsen outcomes by potentiating blood-brain barrier breakdown after injury. We hypothesized that different MMP isoform levels would be predictive of injury severity, secondary injury, and long-term functional outcomes in males and females, respectively. *Methods:* We examined the levels of MMP isoforms in serum samples from a prospective patient biobank (n = 55). Baseline clinical, radiographic, and laboratory data were also analyzed. *Results:* We found that MMP-1 ($P = .036$), MMP-2 ($P = .014$), MMP-3 ($P < .001$), and MMP-9 ($P = .02$) levels gradually increased over time in male patients until 10 DPI. In female patients, we found a different pattern of activation: MMP-8 ($P = .02$) was the only isoform that significantly changed with time, which reached a peak at 3-5 days postinjury. Several MMP isoforms correlated with markers of secondary injury in female patients (all $P < .05$). Additionally, serum levels of MMP-3 ($P = .011$) in males and MMP-10 ($P = .044$) in females were significantly associated with long-term functional outcomes in a sex-specific manner. *Conclusions:* This is the first sex-specific study to examine serum MMP levels and their correlation with clinicoradiologic measures after intracerebral hemorrhage, and identifies potential biomarkers of secondary injury and long-term outcomes in both sexes.

Key Words: Intracerebral hemorrhage—sex differences—matrix metalloproteinases—neuroimaging—clinical research

Background

Intracerebral hemorrhage (ICH) is a major cause of morbidity and mortality worldwide, affecting approximately 2 million individuals each year.[1] Hypertension is the most common cause of spontaneous ICH, accounting for half of brain hemorrhages.[2] In hypertensive ICH, chronic increases in cerebral arterial pressure causes vessels to rupture due to progressive weakening of the vessel wall.[3] Biological sex is an understudied factor for ICH that may influence outcomes, and appears to influence risk in older postmenopausal females.[4] While the overall incidence of ICH is roughly equal in males and females, older females may be more likely to exhibit neurologic deterioration and worse long-term functional outcomes after ICH than males.[5] However, sex-specific data on the clinicoradiologic correlates of these phenotypes are limited.[6] Secondary injury may be driven by elevated blood

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pressure, advanced age, early hematoma expansion, cerebral venous thrombosis, perihematomal edema (PHE), and intraventricular hemorrhage (IVH), all of which could differentially impact male and female ICH patients.[5,7-9]

Altered activity of matrix metalloproteinases (MMPs), a class of proteolytic enzymes that promote vessel rupture during ICH, could mediate poor outcomes in patients by promoting blood-brain barrier breakdown and secondary injury.[10] MMPs are a family of structurally-related zinc peptidases that can be further subdivided based on their extracellular matrix-substrate specificity. These subfamilies include matrilysins (e.g. MMP-7), collagenases (MMP-1 and MMP-8), stromelysins (MMP-3 and MMP-10) and gelatinases (MMP-2 and MMP-9).[10] Previous studies indicate that MMP-2, -3, and -9 increase following ICH in human patients, but were not assessed by patient sex.[10-12] Levels also correlate with various aspects of secondary injury after ICH, including hematoma expansion (MMP-9), PHE (MMP-2 and -9) and early neurologic deterioration (MMP-3).[11-13] While preclinical data suggest an additional role for MMP-9 in mediating Intraventricular hemorrhage (IVH) in preterm neonates, there are a lack of studies examining this relationship in adult patients.[14] Furthermore, while MMPs appear to be useful biomarkers of injury, the relative involvement of various isoforms may be influenced by biological sex.

In preclinical studies, MMP expression has been shown to reduce blood-brain barrier integrity and worsen injury after spinal cord injury[15] and ICH[16] in female mice. An additional study in humans found that MMP-9 levels were stronger predictors of outcome in females with coronary artery disease.[17] Given the well-documented sex differences in pathophysiology and outcomes in preclinical models of ICH and other types of brain injury, there is a need to examine MMPs as specific biomarkers of prognosis after ICH in females. Therefore, we hypothesized that MMP isoforms would differentially predict secondary injury and long-term functional outcomes following ICH in females. To test this hypothesis, we measured serum levels of MMP isoforms, and then correlated them with clinicoradiologic data in male and female ICH patients.

Methods

Study Population

The current study utilized ICH patient data and serum samples from the University of Texas Health Science Center in Houston (n = 55 patients). The Institutional Review Board approved the study, and all patients participating were consented. Patient data were abstracted from prospective ICH databases detailing admission data, radiology, hospital course, and functional outcomes. The study included patients with hypertensive hemorrhagic injury to deep brain structures (basal ganglia and thalamus) as confirmed by an experienced neurologist (N.J.E.). Hemorrhages due to traumatic brain injury, cerebral amyloid angiopathy, or underlying vascular lesions were excluded from the study. For sex-specific analysis of biomarker correlation with injury severity, secondary injury, and functional outcomes, patients were further subdivided into male (n = 39) and female (n = 16) groups based on self-reported sex.

Measurement of Neurological Function and Outcomes

Initial ICH severity was assessed using ICH score, NIH stroke scale, and Glasgow Coma Scale.[18-20] Additionally, functional outcomes were assessed by modified Rankin Scale (mRS) at time of discharge and EuroQol at 90 days postinjury (DPI).[21,22] Finally, discharge status was scored on a 5-point scale: 1 (home), 2 (rehabilitation), 3 (skilled nursing facility), 4 (long-term acute care), and 5 (dead).

Radiologic Measurements

Our primary radiologic outcomes were hematoma volume, PHE, and IVH as quantified via computer-based analysis with Medical Image Processing, Analysis, and Visualization software. IVH severity was estimated on a semiquantitative scale of increasing ventricular involvement developed at UT Health.[23] Hematoma, IVH, and PHE volumes were determined throughout the patient's entire hospitalization via review of every head CT obtained as a part of routine clinical care until discharge.

Serum Sample Collection

Serum samples were obtained from our study cohort at prespecified timepoints postictus: timepoint 1 (0-24 hours), timepoint 2 (24-48 hours); timepoint 3 (3-5 days), timepoint 4 (6-8 days), and timepoint 5 (10 days). Serial samples were collected from each patient when possible, for a total of 136 serum samples. In order to minimize timing bias, all biosamples were timed from the onset of ICH ictus and collected by a biospecimen collection team who were blinded to the clinical status of the patient. Samples were processed within 1 hour of collection and stored at −80°C for future use.

Measurement of Serum Analytes

Samples were thawed on ice for 1 hour and thoroughly vortexed prior to beginning any assays. Serum MMP-1, -2, -3, -7, -8, -9, and -10 were measured using the Bio-Plex Pro Human MMP Panel (Bio-Rad). All assays were performed and read according to the manufacturer's instructions by blinded investigators (MDH and JWF).

Statistical Analysis

All statistical tests were performed by a blinded statistician (L.Z.). Power analysis was performed using the Pearson method.[24] Descriptive statistics (median, interquartile range, frequency, and percentage) were provided for

clinical variables (Table 1). Comparison between male and female patients were performed by using Wilcoxon rank sum test, chi-square test, or Fisher's exact test. Spearman correlation was provided among radiologic variable, clinical variable, and peak cytokine levels (Tables 2-4). *P* values were adjusted for multiple testing on cytokines by Benjamini Hochberg procedure to control the false discovery rate within .1 (denoted as a *q* value).[25] Furthermore, multivariable models were used to confirm the relationship between peak cytokine levels and functional outcomes (Euroqol at 90 DPI), adjusted for initial injury severity (ICH score). Finally, longitudinal mean plot with error bars was provided for each cytokine (Fig 1). Mixed effect models with cytokine level as dependent variable and sex, time, and their interaction as independent variables was used to study the time effect in male and female patients. All statistical analyses were performed in SAS 9.4 software (Cary, NC).

Results

Male and Female Patients Exhibited Differential Expression of MMP Isoforms Over Time

Sex-specific demographic and admission variables are shown in Table 1. Despite similarities in age, race, initial blood pressure, initial neurological function, and hematoma volume, following admission, we observed several statistically significant time effects on MMP expression in each sex (Fig 1). In male patients, we observed significant variation over time in serum MMP-1 ($F[4, 87] = 2.7$, $P = .036$), MMP-2 ($F[4, 87] = 3.3$, $P = .014$), MMP-3 ($F[4, 87] = 5.2$, $P < .001$), and MMP-9 levels ($F[4, 87] = 3.1$, $P = .02$). In contrast, in female patients, we observed significant variation over time in serum MMP-8 levels ($F[4, 87] = 2.64$, $P = .02$), and trending significance in MMP-10 levels ($F[4, 87] = 2.41$, $P = .055$).

Elevated Serum MMPs are Differentially Associated with Clinicoradiologic Markers of Secondary Injury in Female Patients

We next performed a univariate correlation analysis to assess how peak serum levels of MMPs relate to clinicoradiologic measures of secondary injury (hematoma expansion, IVH, and PHE) following ICH. Interestingly, several sex-specific correlations were noted in female patients (Table 2). We found that MMP-2 negatively correlated with the severity of IVH (Table 2, $q = .028$). We also found broad positive associations of MMP-1, -2, -3, and -9 with initial and peak PHE volume in females (see Table 2 for *q* values). However, we found no significant correlation between MMPs and secondary injury in male patients.

Serum MMP Isoforms Selectively Predict Functional Outcomes in Male and Female Patients

We next performed an additional univariate analysis to screen peak levels of serum MMP isoforms as significantly correlated with functional outcomes after ICH at discharge and at 90 DPI. After multiple testing correction, we did not observe significant correlations between MMP levels and short-term functional outcomes (measured as modified Rankin Scale or discharge level: Table 3). We next examined whether serum MMP levels are biomarkers significantly correlated with long-term functional outcomes at 90 DPI in both male and female patients (Table 4). In our combined cohort, we found that elevated MMP-3 ($q = .096$), MMP-7 ($q = .075$), and MMP-10 ($q = .075$) were associated with higher EuroQol scores at 90 DPI, indicating worse functional outcomes. Interestingly, MMP-10 only significantly correlated with functional outcomes in female patients ($q = .048$). We did not observe a significant effect of patient sex on functional outcomes at discharge or 90 DPI.

Table 1. *Patient demographics and admission variables*

Clinical variable		Total cohort n = 55	Male patients n = 39	Female patients n = 16	Significance	
Age, years		56 (13)	56 (14)	56 (13)	$P = .445$	ns
Race	Black	25 (45.5%)	17 (43.6%)	8 (50%)	$P = .544$	ns
	White	20 (36.4%)	16 (41%)	4 (25%)		
	Asian	4 (7.3%)	2 (5.1%)	2 (12.5%)		
	Other	6 (10.9%)	4 (10.3%)	2 (12.5%)		
Systolic blood pressure, mm Hg		203 (52)	202 (67)	206.5 (35.0)	$P = .956$	ns
Diastolic blood pressure, mm Hg		111 (36)	111 (39)	111 (43)	$P = .617$	ns
GCS		11 (7)	11 (6)	11 (7)	$P = .756$	ns
ICH score		2 (1)	1 (1)	2 (1)	$P = .173$	ns
NIHSS		19 (10)	19 (9)	19.5 (9.5)	$P = .605$	ns
Initial hematoma volume, cm^3		16.5 (15.3)	16.7 (15.6)	14.6 (10.3)	$P = .797$	ns

Abbreviations: IH, Intracerebral hemorrhage; NIHSS, NIH stroke scale.

Patients are subdivided into male and female groups. Data are presented as median (IQR). Continuous/ordinal data were analyzed by Wilcoxon rank sum test, and categorical data (race, presence of intraventricular hemorrhage) were analyzed by Fisher's exact test. Significance: ns, no significance.

Table 2. *Serum MMP levels correlate with radiologic markers of injury in female patients*

Radiologic variable		Cytokine	r(s)	Raw Significance		Corrected Significance	
IVH score		MMP-2	−.76	$P = .004$	**	$q = .028$	Yes
		MMP-9	.58	$P = .049$	*	$q = .154$	No
Peak hematoma volume		MMP-1	.58	$P = .029$	*	$q = .103$	No
		MMP-9	.6	$P = .025$	*	$q = .103$	No
PHE	Initial volume	MMP-2	.87	$P < .001$	***	$q < .001$	Yes
		MMP-3	.67	$P = .013$	*	$q = .045$	Yes
	Peak volume	MMP-1	.8	$P = .002$	**	$q = .007$	Yes
		MMP-2	.72	$P = .008$	**	$q = .019$	Yes
		MMP-3	.89	$P < .001$	***	$q < .001$	Yes
		MMP-9	.64	$P = .024$	*	$q = .042$	Yes
	% Increase	MMP-1	.73	$P = .007$	**	$q = .044$	Yes
		MMP-3	.69	$P = .013$	*	$q = .044$	Yes
		MMP-9	.63	$P = .028$	*	$q = .066$	Yes

Abbreviations: IVH, Intraventricular hemorrhage; MMP, matrix metalloproteinas; PHE, perihematomal edema.

No statistically significant correlations were observed in male patients. Data were analyzed by Spearman correlation (n = 12-14 female and 30-34 male patients). Raw significance: * $P < .05$, ** $P < .01$, and *** $P < .001$. Raw P values were adjusted for 7 MMP isoforms to control the false discovery rate (q) for each variable. Statistical significance was defined as $q < .1$.

Table 3. *Association of serum MMP levels with discharge*

Clinical variable	Cytokine	r(s)	Raw significance		Corrected significance	
Female patients						
Discharge mRS	MMP-1	.5	$P = .047$	*	$q = .166$	No
	MMP-9	.53	$P = .037$	*	$q = .166$	No
Discharge Disposition	MMP-8	−.53	$P = .035$	*	$q = .757$	No
	MMP-10	−.51	$P = .045$	*	$q = .757$	No
Male patients						
Discharge mRS	MMP-1	−.02	$P = .915$	ns	$q = .915$	No
	MMP-9	.12	$P = .498$	ns	$q = .871$	No
Discharge Disposition	MMP-8	.15	$P = .375$	ns	$q = .796$	No
	MMP-10	−.05	$P = .76$	ns	$q = .886$	No

Abbreviation: MMP, matrix metalloproteinas.

Data are analyzed by Spearman correlation (n = 16 female and 38-39 male patients). Raw significance: * $P < .05$. Raw P values were adjusted for 7 MMP isoforms to control the false discovery rate (q) for each variable. Statistical significance was defined as $q < .1$.

Table 4. *Serum MMP isoforms predict long-term functional outcomes in a sex-specific manner*

Functional outcomes	Cytokine	r (s)	Raw significance		Corrected significance	
Total cohort						
Euroqol	MMP-3	.39	.041	*	$q = .096$	Yes
	MMP-7	.44	.02	*	$q = .075$	Yes
	MMP-10	.43	.021	*	$q = .075$	Yes
Female patients						
Euroqol	MMP-10	.89	.007	**	$q = .048$	Yes
Male patients						
Euroqol	MMP-3	.5	.021	*	$q = .113$	No
	MMP-7	.43	.032	*	$q = .113$	No

Abbreviation: MMP, matrix metalloproteinas.

Data are analyzed by Spearman correlation (n = 7 female and 21 male patients). Raw P values were adjusted for 7 MMP isoforms to control the false discovery rate (q) for each variable. Raw statistical significance was defined as * $P < .05$ and ** $P < .01$. Corrected statistical significance was defined as $q < .1$.

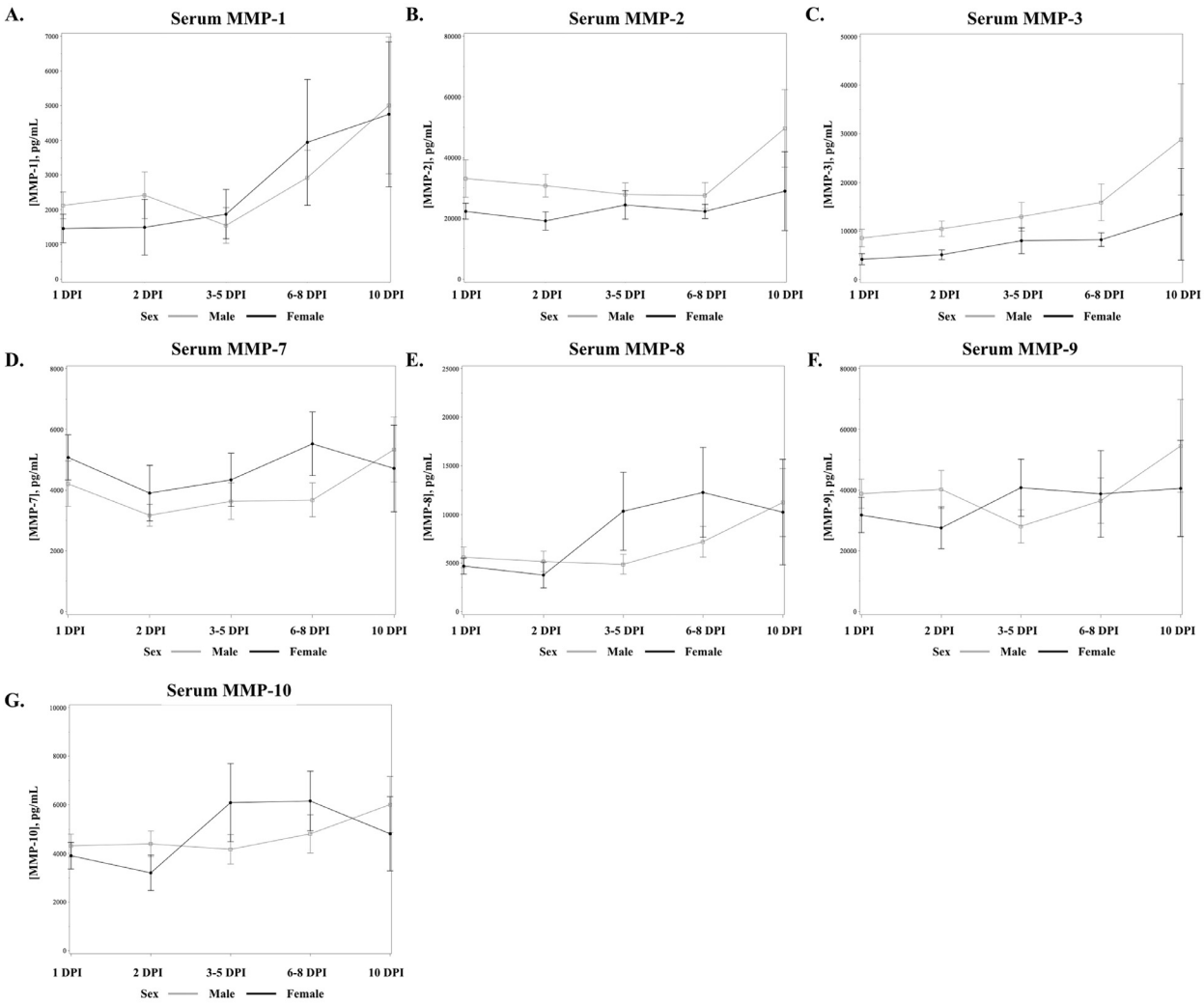

Figure 1. *Time-course of serum MMP levels in male and female ICH patients. Data are analyzed using mixed effect models, and presented as mean ± SEM. DPI = days post-injury. 1 DPI, n = 16 female and 37 male; 2 DPI, n = 8 female and 21 male; 3-5 DPI, n = 8 female and 21 male; 6-8 DPI, n = 6 female and 14 male; 10 DPI, n = 5 female and 12 male.*

Finally, we confirmed the sex-specific utility of MMP-3, -7, and -10 in predicting long-term functional outcomes (assessed by EuroQol score) by performing multivariate regression to control for the impact of ICH severity (quantified by ICH score) on functional outcomes at 90 DPI. In all patients, we found that MMP-3 ($P = .011$) and MMP-7 ($P = .044$) significantly associated with outcomes, with trending significance observed in MMP-10 ($P = .059$). In males, we found that MMP-3 ($P = .016$), but not MMP-7 ($P = .087$) were independently associated with functional outcomes. In females, we confirmed that MMP-10 ($P = .009$) was significantly associated with functional outcomes.

Discussion

In summary, the present study found that (1) males and females exhibited significantly different patterns of MMP expression in the days following ICH: (2) MMPs were more reliable predictors of secondary injury in females compared to males; and (3) MMP-3 and MMP-10 are sex-specific predictors of long-term functional outcomes in male and female patients, respectively. To our knowledge, this is the first sex-specific exploration of serum MMP levels and their correlation with clinicoradiologic measures after spontaneous ICH. While these biomarkers could be clinically translated to improve care for ICH patients, larger studies will ultimately be required to confirm our findings.

Our study newly examined the levels of several MMP isoforms at multiple time-points up to 10 DPI. Both preclinical and human studies have examined the expression of MMP-2, MMP-3, and MMP-9 levels after ICH.[11,13,26,27] However, to our knowledge, ours is the first to characterize the levels of MMP-1, MMP-7, MMP-8, and MMP-10 after spontaneous hypertensive ICH in humans. We found that MMP-1, -2, -3, and -9 levels gradually increased over time in male patients until 10 DPI. In

female patients, we found a different pattern of activation: MMP-8 and MMP-10 (trending) were the only isoforms that changed with time, which reached their peak at 3-5 DPI. Among the enzymes that changed significantly in males, the gelatinases MMP-2 and MMP-9 are some of the best-described biomarkers of ICH injury severity, as they play a major role in degrading the blood-brain barrier and expanding hematoma volume.[11] However, we found that the stromelysins (MMP-3 and MMP-10) were superior predictors of functional outcome in our patient populations, and also displayed a degree of sex specificity. MMP-3 was a significant predictor of long-term outcomes in male patients, but MMP-10 was a significantly stronger predictor of outcome in females. This is interesting, as both stromelysins degrade proteoglycans and fibronectin, implying a major role in connective tissue remodeling after injury.[10] While other studies have shown sex-specific activation of stromelysins, the underlying biological mechanism is not known.[28-30] Future studies associating serum biomarkers with long-term outcomes after ICH should take patient sex into account to improve translation to broader clinical use.

We also examined the association of MMPs with secondary injury (hematoma expansion, PHE, and IVH). Previous work has found that MMP-9 is positively associated with PHE in patients with spontaneous ICH.[11] While we did not observe significant association of MMP levels and secondary injury in male patients, we found multiple novel associations in females. Elevated levels of MMP-1, -2, -3, and -9 were directly associated with various phases of expanding PHE in females. Unexpectedly, high serum MMP-2 was *inversely* associated with IVH severity in females—this interesting finding raises new questions with respect to the role of MMPs in the pathophysiology of IVH. While IVH was previously thought to occur secondary to hematoma expansion, driven in part by the activity of MMPs, our data suggest that higher MMP-2 levels may be protective against IVH in female patients. Further work in preclinical models (such as aged female mice) would be helpful to evaluate the role of MMP-2 as a potential neuroprotective molecule.

While the current study has several strengths, including the stratification of patients by sex, the simultaneous and longitudinal measurement of multiple MMP isoforms, the examination of multiple clinicoradiologic markers of each endpoint, and the correlation with long-term outcome data, it is not without some weaknesses. Although the overall cohort was moderately sized, the number of female patients in our study was relatively small. Coupled with the number of cytokines examined (7 in total), these factors reduced the statistical power and may have led to under-reporting of significant associations in females. This was a particular challenge in assessing long-term functional outcomes (Table 4), as many patients were lost to follow-up and excluded from the analysis. Post hoc power analysis demonstrated that, with 90-day outcomes data on a total of 30 patients, we had a power of 80% to test whether a correlation coefficient as low as .49 differs from zero. For the sex-specific univariate analysis, we had a power to detect a correlation of .56 in males and .87 in females. Despite this relatively low power, statistical significance was attained by Spearman correlation analysis, which may be due to the fact that it is a more sensitive test compared to the Pearson correlation.[24] These results were further confirmed with correction for the false-discovery rate, and also survived subsequent multivariable correction. However, with this in mind, it is clear that there is a need for larger studies to further evaluate and confirm our findings, as there are likely other associations with MMPs that failed to reach significance due to these limitations.

We employed 3 strategies to maximize our statistical power. First, in our univariate analysis, we utilized peak serum values instead of examining all 5 collection time-points for significant associations, which would have dramatically increased the number of comparisons. Second, we preselected a higher FDR threshold ($q < .1$), which is well within the range used in previous studies (ranging from $q = .05$ to $q = .2$) and increased our ability to report significant associations.[25] Third, we also reported both P values and corrected q values, which may aid in designing follow-up studies to examine significant associations that failed to overcome the FDR threshold in our study.

Overall, the current study identified several MMP isoforms that appear to uniquely impact aspects of ICH injury in female patients, and are deserving of further study in both preclinical models and in larger cohorts of patients with spontaneous hypertensive ICH. Our additional interest is to expand these findings into other types of ICH, including lobar and subarachnoid hemorrhage, which may also show a sexually dimorphic disease course.[31,32] Furthermore, the potential interactions of gender identity, socioeconomic status and racial/cultural factors on outcomes after ICH should also be explored in greater detail. A better understanding of the impact of biopsychosocial factors on the pathogenesis of ICH may lead to more selective biomarkers, and ultimately better care, for all ICH patients.

Authors' contributions

M.D.H., L.D.M. and N.R.G. wrote the manuscript. M.D.H. and J.W.F. performed all biochemical assays. M.D.H. and N.J.E. compiled clinical data. L.Z. performed all statistical analysis.

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Supplementary Materials

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