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Original research article

Peripheral glutamate and TNF-α levels in patients with intracerebral hemorrhage: Their prognostic values and interactions toward the formation of the edemal volume

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ABSTRACT

Objective: We aimed to evaluate the prognostic values, contribution and interactions of the peripheral blood plasma glutamate and tumor-necrosis factor-α (TNF-α) levels toward the formation of the perifocal edema in patients with intracerebral hemorrhage (ICH).
Methods: Fifty patients with ICH and fifty healthy controls were included in the study. The peripheral markers were detected by high-sensitivity ELISA.
Results: A highly significant differences in plasma glutamate and TNF-α levels with good separation of their values was detected between patients and healthy controls. The two variables correlated with the severity of the symptoms and the initial volume of the ICH at admission. Both peripheral glutamate and TNF-α levels at admission were estimated as significant predictors for the formation of the perifocal edema five days after ICH; nevertheless, it was shown that they independently contribute to the development of the edema, without effects of interaction and regardless the localization of the ICH.
Conclusions: Our results support the idea for the significance of glutamate and TNF-α as peripheral markers for excitotoxicity and inflammation in ICH patients. The developed multiple regression model for prediction of the development of the edema could be beneficial in decision making between conservative treatment and surgical intervention in the clinical practice.

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1. Introduction

Intracerebral hemorrhage (ICH) accounts for 10–15% of strokes [1]. It is the deadliest subtype of stroke associated with worse neurological outcome when compared to the ischemic stroke, with mortality rate of 37–52% after 30 days [2] and in-hospital mortality of 40% [3].

The biggest neurological deterioration after ICH occurs due to the formation of the perihematomal edema, a proven significant predictor for bad neurological outcome [4]. The long-standing dilemma whether patients should be treated with conservative treatment or surgical intervention was a matter of subject years ago [5]; nevertheless, the results from the international STICH study have shown no overall benefit from early surgery when compared to the initial conservative treatment [6], leaving the clinicians with the same problem of decision making without any obvious direction. Therefore, the search for a molecular marker that could predict the formation of the edema is a major challenge, because these patients can be treated in advance with potent anti-edematous therapy or early craniotomy, thus preventing neurological deteriorations and future bad outcome.

In the last several years, the focus of the interests for intervention in ICH has slowly shifted from the acute to the post-hemorrhagic phase [7]. Several animal models for experimental ICH have been developed [8,9], and the pre-clinical studies using these models have revealed that the secondary brain injury after ICH is mainly triggered by inflammatory mechanisms and formation of peri-hematomal edema [10–12]. All of these studies have reported increased levels of pro-inflammatory mediators immediately after the bleeding [13,14], with the tumor necrosis factor-α (TNF-α) being argued as one of the major constituents of this process [7]. On the other side, contrary to the ischemic stroke, the contribution of excitotoxicity in the development of brain injury after ICH has not been well defined [15]. Several studies have shown higher glutamate levels in the brain of experimental animals with induced ICH [16] and in the perihematomal area in patients with deep ICH [17], suggesting that excitotoxicity may also contribute to the secondary brain injury. However, their interaction with the other primary mechanisms of brain injury has not been studied in details.

A vast number of pre-clinical studies have shown the effect of TNF-α on the secondary brain injury in animal models of ICH, but however detailed clinical studies that evaluate their role in patients are very rare. An increased variability of the blood–brain barrier (BBB) has been reported after ICH [18], leading to the hypothesis that the excitotoxic and pro-inflammatory mediators can transfer from the brain in the blood and be detected peripherally. The fact that plasma glutamate concentration can reflect the released glutamate levels from the brain tissue in patients with ischemic stroke [19] supports this hypothesis.

The aim of the present study is to evaluate the role of peripheral plasma glutamate and TNF-α levels as biomarkers for ICH, in respect to the anatomic localization of ICH, the hemispheric side, the symptom severity and the initial volume of ICH, and moreover, to examine the prognostic role and possible interactions of these variables on the development of the volume of the edema five days after ICH.

2. Methods

2.1. Subjects and study design

We have included 50 patients with acute, primary, supratentorial ICH, recruited from the University clinics of Neurosurgery and Neurology and 50 healthy controls in the period from 01.01.2014 to 31.10.2016. The study was approved by the Ethical committee and all subjects have given informed consent.

For the purposes of the initial screening, all subjects were called for a short conversation and their medical history as well as basic demographic characteristics were recorded in a medical questionnaire. The inclusion criteria for entering the study were absence of any medical history or other neurological, neurodegenerative or psychiatric disorders, as well as absence of any medical history of cardiovascular, pulmonary, renal and hepatic diseases, coagulopathies and any inflammatory conditions or immune diseases that could influence TNF-α levels. The time interval between the onset of ICH and the admission was less than 24 h in all patients. A detailed neurological examination was performed on every patient by a team of experienced neurologist and neurosurgeon. The severity of the symptoms was estimated according to the Canadian Stroke Scale (CSS).

Controls underwent detailed interview to exclude presence of current or any past history of neurological, neurodegenerative and mental diseases. Healthy controls were selected to match patients according to gender and age, so that the effects of these variables are excluded (Table 1).

2.2. Radiological analyses

Two CT scans were performed on all patients: at admission and five days after ICH. The volume of the hematoma was measured by performing transversal slices with slice thickness of 3.5–10 mm. The calculations of the volume of the hematoma and the edema were performed according to the ABC/2 formula, as described before [20]. Several patients had irregular ICH volume and in this case, we have used the calculator for irregular volumes [21,22]. The edema was defined as the most hypodense area immediately surrounding the ICH and more hypodense than the corresponding area in the contralateral hemisphere, according to the present recommendations [23] and it was determined by subtracting the volume of the ICH from that of the total lesion, as performed in the previous studies [24,25] (given as supplementary material).

2.3. Biochemical analyses

At admission, blood was collected in the morning (after overnight fasting) in EDTA-anticoagulated tubes. The tubes were centrifuged at 3,000 rpm, 15 min at +4 °C. The obtained blood plasma was aliquoted and stored at −80 °C till further analyses. The quantitative detection of glutamate and TNF-α was performed using the ELISA kits from Abnova (Glutamate ELISA Kit, KA1909) and Quantikine (Human TNF-alpha Quantikine ELISA Kit, DTA00C), according to the manufac-

Table 1 – Baseline characteristics of patients and healthy controls.

Variables	Healthy controls	Patients	*p*-value
Number of subjects	50	50	
Gender			
Male	25 (50%)	25 (50%)	n.s.[a]
Female	25 (50%)	25 (50%)	n.s.[a]
Age			
Less than 60 years	12 (24%)	9 (18%)	n.s.[a]
60–70 years	25 (50%)	21 (42%)	n.s.[a]
71–80 years	9 (18%)	13 (26%)	n.s.[a]
More than 80 years	4 (8)	7 (14%)	n.s.[a]
Systolic blood pressure (mmHg)	125 (IQR = 10)	175 (IQR = 11)	0.02[b]
Diastolic blood pressure (mmHg)	70 (IQR = 8)	100 (IQR = 11)	0.03[b]
Blood glucose levels (mmol/L)	4.3 (IQR = 1.5)	7.6 (IQR = 1.7)	0.002[b]
CSS score at admission	–	7.0 (IQR = 4.5)	–
Anatomic localization of ICH			
Lobar	–	15 (30%)	–
Deep (basal ganglia)	–	35 (70%)	–
Hemispheric side			
Left	–	26 (52%)	–
Right	–	24 (48%)	–
Initial volume of ICH (cm^3)	–	25.1 (IQR = 42.3)	–
Volume of the perifocal edema after 5 days (cm^3)	–	33.8 (IQR = 48.9)	–

[a] Calculated with contingency table and χ^2.
[b] Calculated with Mann–Whitney *U* test.

turer's instructions. The blood plasma glucose levels were measured with according to the GLUCOSE GOD/PAP kit from Biosystems.

2.4. Statistical analyses

The statistical analyses were performed using both the statistical software IBM SPSS Statistics 21 and Statistica 7 (StatSoft©). Categorical variables are expressed as number of subjects and %. Since the distribution of the values of all continuous variables differed significantly from normality, these results are expressed as median and interquartile range (IQR). The Mann–Whitney *U*-test was used to compare the differences in the central tendencies between the groups, and the non-parametric Levene test was used to test the differences in the variances. Bivariate statistical analyses were performed using non-parametric correlation with Spearman coefficient. The multiple regression model was performed after logarithmic transformation of the values. A moderation analysis and two-way ANOVA was used for estimating effects of interaction. In all cases, the level of statistical significance was defined as $p < 0.050$, i.e. $p < 0.001$ for highly significant.

3. Results

3.1. Blood plasma glutamate and TNF-α levels between patients with ICH and healthy controls

Median blood plasma glutamate levels were significantly higher in patients with ICH, when compared to healthy controls ($U = 26.0$, $p = 3.2 \times 10^{-17}$; Fig. 1, panel a). Patients with ICH had also significantly higher levels of median blood

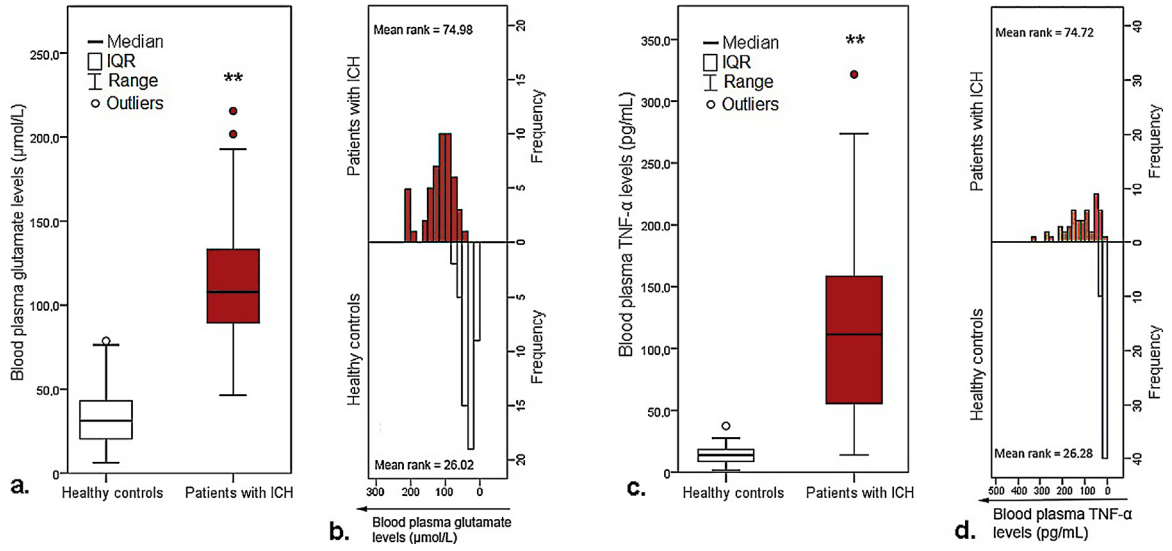

Fig. 1 – Peripheral blood plasma glutamate and TNF-α levels between patients with ICH and healthy controls. (a) Comparison of median blood plasma glutamate levels. (b) Histogram of the distribution of the values for glutamate between patients and healthy controls. A good separation of the values can be observed. (c) Comparison of median blood plasma TNF-α levels. (d) Histogram of the distribution of the values for TNF-α between patients and healthy controls. The good separation of the values is evident. **$p < 0.001$.

plasma TNF-α levels ($U = 39.0$, $p = 9.9 \times 10^{-17}$; Fig. 1, panel c). We have observed very good separations of the values for glutamate (Fig. 1, panel b) and especially TNF-α (panel d) between the two groups.

Nevertheless, we have detected higher variability of the glutamate and TNF-α values within the patient group. Patients had 10 times bigger variability of the TNF-α levels ($F = 79.7$, $p = 2.58 \times 10^{-14}$). The differences in the IQR of the glutamate levels were not that astounding, but were also proven statistically significant ($F = 19.7$, $p = 0.00002$). Both plasma glutamate and TNF-α levels within the patient group did not differ significantly between males and females ($U = 206.0$, $p = 0.097$ and $U = 275.5$, $p = 0.473$ respectively), nor between the different age groups ($\chi^2 = 4.382$, $p = 0.289$ and $\chi^2 = 5.167$, $p = 0.160$). Also, we did not detect any significant differences in the glutamate and TNF-α levels in patients with lobar and deep ICH ($U = 253.0$, $p = 0.841$ and $U = 228.0$, $p = 0.465$ respectively), as well as between patients that had ICH on the right or the left hemisphere ($U = 277.0$, $p = 0.646$ and $U = 284.5$, $p = 0.593$).

A negative and low correlation between the CSS scores for symptom severity at admission and plasma glutamate levels in ICH patients has been detected ($\rho = -0.331$; $p = 0.019$). The correlation between the CSS scores and plasma TNF-α levels in ICH patients was negative, moderate and statistically highly significant ($\rho = -0.567$; $p = 0.00002$). We have also detected positive and moderate correlation between blood plasma glutamate levels and the initial volume of ICH at admission ($\rho = 0.453$, $p = 0.00001$). The correlation between the plasma TNF-α levels and the initial volume of ICH was even higher ($\rho = 0.653$, $p = 2.7 \times 10^{-7}$), suggesting that both of these variables are influenced by the initial volume of the hematoma.

3.2. *Effects of the biochemical variables on the development of the perifocal edema after 5 days*

The results from the multiple regression model for the influence of the followed variables on admission on the development of the perifocal edema five days after ICH are shown in Table 2.

Blood plasma glutamate and TNF-α levels as well as the initial volume of ICH were estimated as the only significant predictors that influence the development of the perifocal edema. According to the standardized β coefficients, it seems evident that the TNF-α levels have the strongest effect on the volume of the edema.

The following equation for estimation of the perifocal edema can be generated from the model:

$$V(\text{perifocal edema, 5th day}) = -1.424 + 0.423 \cdot c(\text{TNF-}\alpha \text{ at admission}) + 0.232 \cdot c(\text{glutamate at admission}) + 0.409 \cdot V(\text{ICH at admission})$$

The predictive capacity of the generated model was estimated as very high ($R^2 = 0.753$, adjusted $R^2 = 0.736$, standard error of estimate = 0.243) and with high degree of significance (ANOVA $F = 46{,}626$; $p = 5.4 \times 10^{-14}$).

Bearing in mind that the main focus of this paper is the effect of the biochemical variables and their possible role as predictors for the development of the perifocal edema, we have also performed a second analysis where the clinical and radiological variables were omitted. These results are represented on the next table (Table 3).

The predictive capacity of the second generated model was slightly lower, but still it was estimated as high ($R^2 = 0.654$, adjusted $R^2 = 0.639$, standard error of estimate = 0.284), with high degree of significance (ANOVA $F = 44.350$, $p = 1.5 \times 10^{-11}$). According to the second model, once again the gender, the anatomic localization of ICH, the hemispheric side with ICH, the systolic blood pressure, the diastolic blood pressure and the blood glucose levels were excluded as non-significant predictors for the development of the edema, However, the effects of the TNF-α levels and glutamate were confirmed as significant predictors, especially stressing the effect of the TNF-α levels. The results from the proposed regression analyses were modeled and depicted in Fig. 2.

3.3. *Effects of interaction between blood plasma glutamate and TNF-α levels on the volume of the perifocal edema five days after ICH*

The correlation between plasma glutamate and TNF-α at admission in our patients with ICH was positive, low and

Table 2 – Multiple regression model for the development of the perifocal edema five days after ICH. The values were log transformed. Statistically significant predictors are given in bold.

Independent predictors	Standardized β coefficients	p-value
Gender	−0.051	0.525
Anatomic localization of ICH	−0.062	0.408
Hemispheric side	0.001	0.989
Systolic blood pressure (mmHg)	0.056	0.460
Diastolic blood pressure (mmHg)	0.089	0.259
Blood glucose levels (mmol/L)	0.043	0.394
Blood plasma glutamate levels (μmol/L)	**0.232**	**0.007**
Blood plasma TNF-α levels (pg/mL)	**0.423**	**0.00003**
CSS score at admission	0.091	0.295
Initial volume of ICH at admission	**0.409**	**0.00009**

Table 3 – Multiple regression model for the development of the perifocal edema five days after ICH (clinical and radiological variables are omitted). The values were log transformed. Statistically significant predictors are given in bold.

Independent predictors	Standardized β coefficients	p-value
Gender	−0.054	0.872
Anatomic localization of ICH	−0.101	0.985
Hemispheric side with ICH	−0.062	0.483
Systolic blood pressure (mmHg)	0.089	0.315
Diastolic blood pressure (mmHg)	0.093	0.315
Blood glucose levels (mmol/L)	0.011	0.899
Blood plasma TNF-α levels (pg/mL)	**0.627**	**1.3×10^{-8}**
Blood plasma glutamate levels (μmol/L)	**0.339**	**0.001**

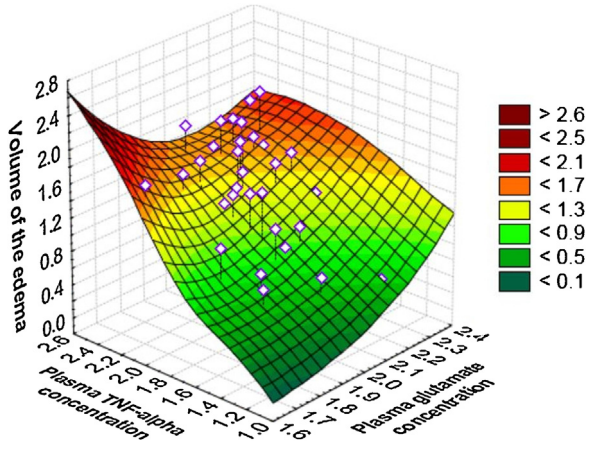

Fig. 2 – Effects of blood plasma TNF-α and glutamate levels on the volume of the perifocal edema five days after ICH. The values were log transformed.

significant ($\rho = 0.339$, $p = 0.004$). The linear regression model for the relation between these two variables has also been given a low support ($R^2 = 0.132$; Fig. 3, panel a). However, to test the effects of their possible mutual interaction, we have performed moderation analyses by multiplying the glutamate and TNF-α levels. The results are represented in Table 4.

The multiplied TNF-α × glutamate variable was not detected as a significant predictor for the development of the perifocal edema, which indicates absence of any interaction between these variables.

Additionally, we have also performed two-way ANOVA analyses. On the basis of the median values, the patients were coded into two groups: patient subgroup with low and patient subgroup with high glutamate and TNF-α levels. Then, the effects of interaction of these variables were analyzed (low or high levels) on the volume of the perifocal edema. This analysis again confirmed absence of any significant interaction ($F = 0.000$, $p = 0.999$, Fig. 3, panel b).

4. Discussion

The detrimental effects of the excitotoxicity in the ischemic stroke were a central subject for examination long years ago [26], but the actual contribution of the glutamate in the brain damage following hemorrhagic stroke started to be examined in the last 10 years, with more or less contradictory findings in spite with the other proposed mechanisms that were commonly reported as primary triggers [27,28]. The first report for increased glutamate levels after ICH have been gained using in vivo brain microdialysis in experimental animals with induced ICH [16]. The authors in this study have detected 4 times higher glutamate levels ipsilateral to the hematoma 30 min after ICH that remained increased in the next 5 h. However, only several studies have explored the role of the blockade of the glutamate accumulation on brain injury after ICH. Mendelow [29] has shown that D-CPP antagonist on the NMDAR receptor can reduce the volume of the edema in experimental animals. Moreover, it have also been reported that the non-competitive antagonist of the NMDA receptor, memantine, can reduce the expansion of the hematoma, the cell death and the infiltration of immune cells [30].

Contrarily, the effects of TNF-α after ICH are well documented. Recently, Behrouz [7] proposed a model for the contribution of TNF-α on edema formation, based on the previous results that the production of thrombin after ICH triggers increase of the TNF-α brain levels [18]. It seems that the increased TNF-α levels activate the receptors on the microglia cells and astrocytes and are also involved in the regulation of the permeability of the blood–brain barrier and the glutaminergic transmission [31].

In the present study, we have shown higher blood plasma glutamate and TNF-α levels in patients, when compared to

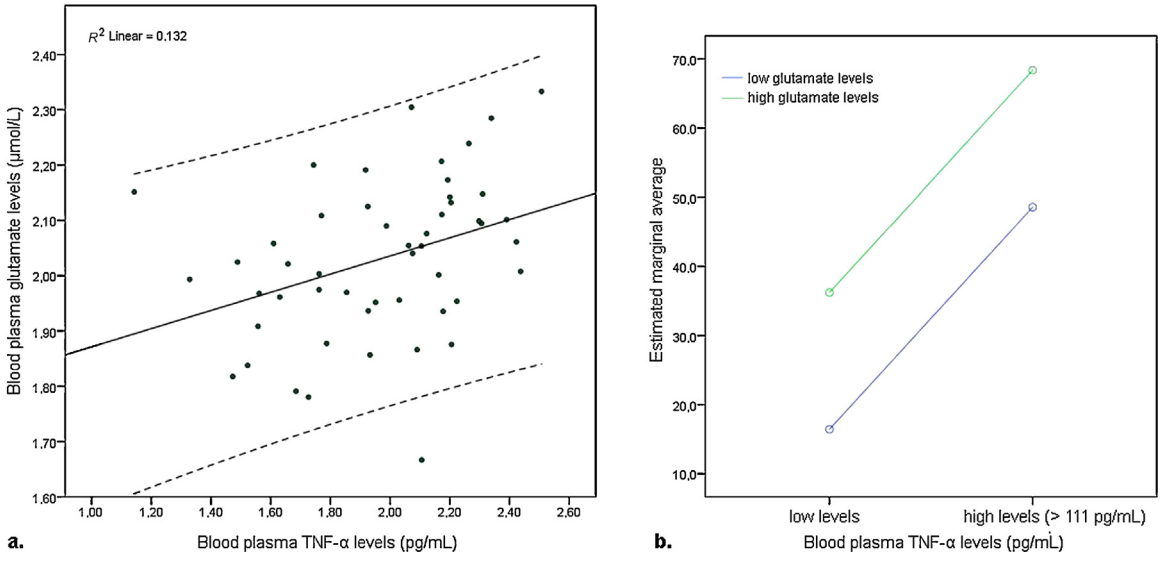

Fig. 3 – Effects of interaction between blood plasma glutamate and TNF-α levels on the volume of the perifocal edema five days after ICH. (a). Linear regression model for the relations between blood plasma glutamate and TNF-α levels (log transformed). (b) Two-way ANOVA for estimating effects of interaction.

Table 4 – Moderation analysis for the effects of interaction between glutamate and TNF-α on the volume of the perifocal edema. Statistically significant predictors are given in bold.

Independent predictors	Standardized β coefficients	p-value
Blood plasma TNF-α levels (pg/mL)	**2.624**	**0.020**
Blood plasma glutamate levels (μmol/L)	**0.884**	**0.001**
Blood plasma glutamate × TNF-α levels	−2.246	0.152

healthy controls. It seems that these differences reflect the brain abnormalities detected in animal models for ICH, most probably because of the increased permeability of the blood–brain barrier and their influx in the blood. The good separation of the values between patients and healthy controls can clearly support the idea for their significance as peripheral markers for excitotoxicity and inflammation in ICH patients.

However, only several studies have examined the peripheral blood levels of these two mediators in patients with ICH. Hitherto, increased TNF-α levels at admission were detected as predictors for increased mortality in patients at intensive care [32]. High peripheral TNF-α levels were also reported as a predictor for early hematoma growth and were associated with increased risk for mortality and bad neurological outcome after three months in patients with ICH [33]. Domac et al. [34] have detected higher TNF-α levels in patients with acute ischemic stroke, when compared to healthy controls. On the basis of these findings, Maas and Furie [35] have listed TNF-α as a molecular biomarker that cannot serve for distinction between ischemic and hemorrhagic stroke and as a marker not useful for early diagnosis of ICH but potentially useful for its subacute diagnosis. Yang and Shao [36] have detected higher levels of TNF-α in patients with hypertensive ICH at admission, when compared with healthy controls, with a peak of the TNF-α concentration three days after ICH. However, the studies for peripheral blood levels of glutamate in patients with ICH are even scarcer; in this context Castillo et al. [25] have revealed significant differences in glutamate levels between patients with good and bad neurological outcome, but this study lacks any comparison of these values with healthy controls.

We have also detected higher variability of glutamate and TNF-α values within the patient group, but however we have not revealed any significant effects of the gender, age, the anatomic localization of ICH and the hemispheric side on this variability. Nevertheless, glutamate and TNF-α values correlated with symptom severity and the initial volume of ICH. These findings are in a good agreement with several studies [25,36]. In our study, we have also assumed possible contribution of the anatomic localization of ICH on the peripheral levels of these two biochemical variables. However, it was shown absence of any effect of the anatomic localization of ICH on plasma glutamate and TNF-α levels. In line with these findings, it seems that the symptom severity and the initial volume of ICH are the major drivers for the variability of the glutamate and TNF-α levels in patients with ICH.

Concerning the effects of the clinical, biochemical and CT variables on the volume of the perifocal edema, our developed multiple regression model has revealed that the initial volume of ICH, and both plasma glutamate and TNF-α levels are significant predictors that induce the development of the perifocal edema. This result is not in an agreement with some previous studies where TNF-α was estimated as the only significant biomarker for the volume of the perifocal edema [25]. Nevertheless, the authors in the same paper show positive correlation between blood plasma glutamate levels and the volume of the edema, suggesting that both excitotoxicity and inflammation contribute to the secondary brain injury. In our study, we have shown absence of any effect of interaction between these two mechanisms, which indicates that both glutamate and TNF-α individually contribute to the volume of the perifocal edema, with TNF-α being the strongest predictor for its development. Once again, the anatomic localization of ICH was not estimated as significant predictor for the formation of the edema.

These findings are in very good congruence with the previously listed results from the animal models for ICH that suggest possible contribution of glutamate and TNF-α in the formation of the edema. For instance, Wu et al. [37] have detected that perihematomal glutamate increased significantly after ICH and a strong association between these high levels with BBB disruption and brain edema was detected, suggesting that glutamate may play an important role in the secondary brain injury. Two studies have also reported decreased levels of perihematomal glutamate levels after minimally invasive procedure for ICH evacuation in animal models [38,39]. In animal models, increased levels of TNF-α were detected in the perihematomal area that contribute to the development of the edema via increased BBB permeability [40]. Bearing in mind the high predictive capacity of the developed model, we propose that it can be used in future for prediction of the volume of the perifocal edema after ICH which, on the other side, can help in the dilemma between conservative treatment and surgical intervention.

5. Conclusions

In all, our results have shown that patients with ICH have significantly higher blood plasma glutamate and TNF-α levels at admission, when compared to healthy controls, which stresses the importance of these mediators in the pathology of the ICH. The anatomic localization of ICH (lobar/deep; left/right hemisphere) does not influence the influx of glutamate and TNF-α and equally induces excitotoxicity and inflammation, regardless ICH position.

The only significant predictors for the formation of the perifocal edema five days after ICH are the initial volume of the ICH, the peripheral glutamate and TNF-α levels that independently contribute (without any effect of interaction) to the development of the edema, regardless the localization of the ICH. These variables should be considered for prediction of the volume of the perifocal edema after ICH which, on the other side, can help in the dilemma between conservative treatment and surgical intervention. The most powerful predictor for the development of the edema are the TNF-α levels, which points

to the fact that the inflammatory mechanisms have the primary role for the formation of the edema and the secondary brain injury. We believe that these results can contribute in the prevention of the formation of big edema in patients with ICH, as well as in the future development of different therapeutic strategies via blockade of the glutamate and TNF-α receptors and thus prevent their detrimental effects.

Conflict of interest

None declared.

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None declared.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pjnns.2017.10.003.

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