# NEUROINFLAMMATION AS A TARGET FOR INTERVENTION IN SUBARACHNOID HEMORRHAGE

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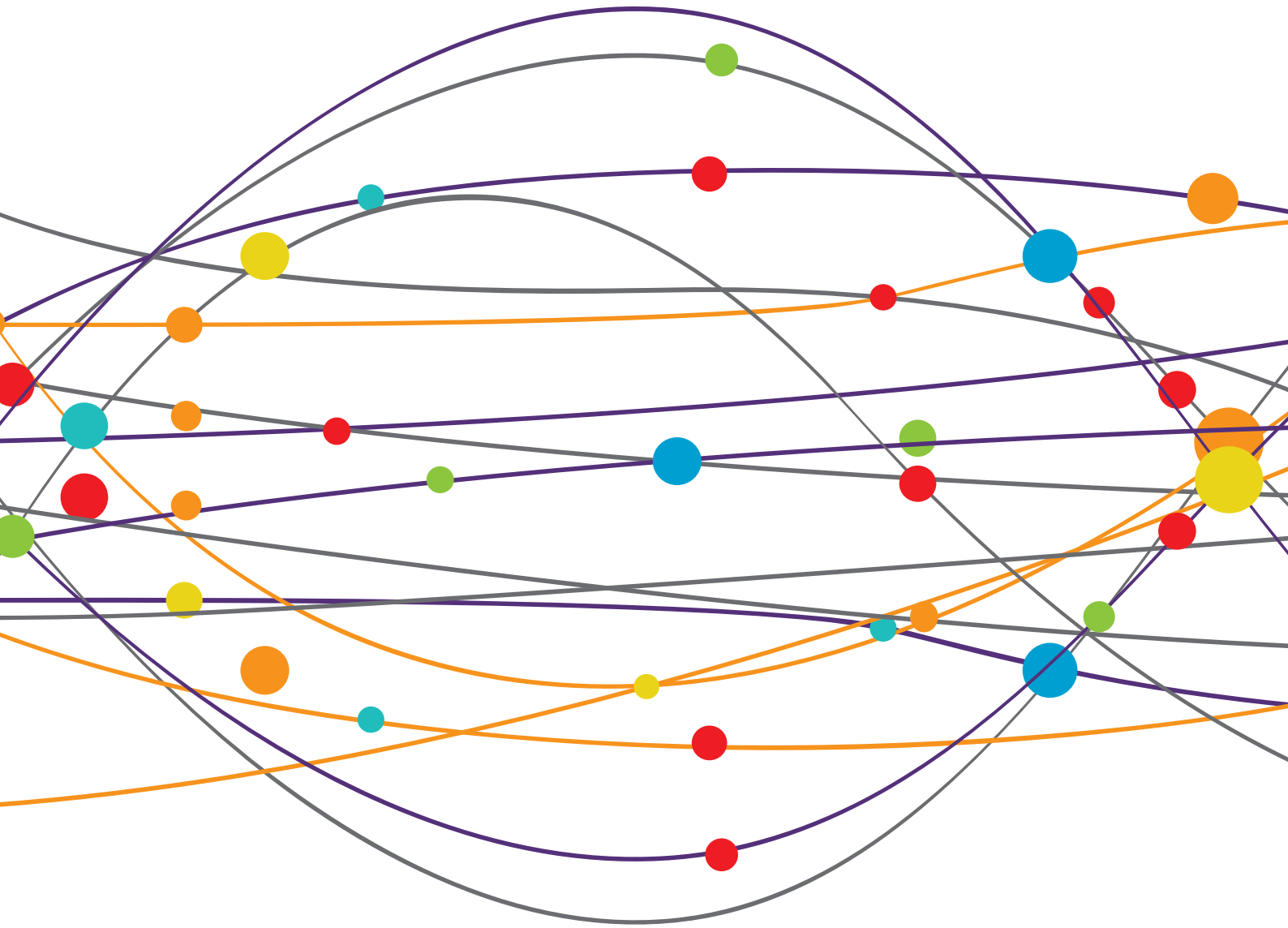

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# NEUROINFLAMMATION AS A TARGET FOR INTERVENTION IN SUBARACHNOID HEMORRHAGE

Topic Editors:
**Fernando Testai,** University of Illinois at Chicago, United States
**J. Marc Simard,** University of Maryland, Baltimore, United States

Aneurysmal subarachnoid hemorrhage (SAH) is a stroke subtype that affects, preponderantly, young adults. This condition carries a mortality of approximately 30-50% and a rate of permanent neurological disability of 30%. In addition, a substantial number of patients with an apparent good outcome suffer from residual neurocognitive impairment which, though subtle, prevents them from returning to work and having a normal life. Based on these data, it has been estimated that SAH is responsible for almost a quarter of all the years lost because of stroke. The calcium channel blocker nimodipine remains the only pharmacological treatment for SAH. This drug, however, has limited effectiveness and its use in clinical practice may be limited due to hypotension. Therefore, novel and effective treatments for this condition are desperately needed. The outcome in SAH has been associated with early brain injury, vasospasm, and delayed cerebral ischemia. Mechanistically, these processes are characterized by micro- and macro-vascular dysfunction, microthrombi formation, blood-brain barrier (BBB) dysregulation, brain edema, and neural-cell survival. Soon after SAH, there is a robust inflammatory response characterized by pyrexia, leukocytosis, and upregulation of adhesion molecules and cytokines in the periphery and in the CNS. Observational studies have shown that patients with more severe inflammatory responses experience worse outcomes after SAH. At the molecular level, different proinflammatory intracellular signaling pathways, including mitogen-activated protein-kinase and nuclear factor kappa-β, are activated in cerebral vessels after experimental SAH and their inhibition has been shown to decrease the occurrence of vasospasm. In addition, clinical and preclinical data have linked cytokine upregulation (interleukins [IL]-1B, IL-6 and IL-8, tumor necrosis factor-α, and monocyte chemoattractant protein-1), enhanced expression of adhesion molecules (selectins, integrins, and ICAM), and neutrophil activation to vasospasm of large cerebral arteries, microvascular dysregulation, and cell death. Moreover, immune cells regulate hemostasis and secrete active proteases, including matrix metalloproteinase 9, which promote microthrombosis and induce blood brain barrier dysfunction, respectively. In this context, it has been suggested that an enhanced inflammatory burden might contribute to brain injury in SAH through numerous downstream mechanisms. On the other hand, growing evidence demonstrates that neuroinflammation may influence the proliferation and migration of progenitor cells that participate of synaptic plasticity, neurogenesis, and neurorepair.

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# Plasma Soluble Urokinase-Type Plasminogen Activator Receptor Is Not Associated with Neurological Outcome in Patients with Aneurysmal Subarachnoid Hemorrhage

*Heikki Kiiski[1]*†, Ville Jalkanen[1†], Marika Ala-Peijari[1], Mari Hämäläinen[2], Eeva Moilanen[2], Jukka Peltola[3] and Jyrki Tenhunen[1,4]*

[1] Critical Care Medicine Research Group, Department of Intensive Care, Tampere University Hospital, Tampere, Finland, [2] The Immunopharmacology Research Group, Faculty of Medicine and Life Sciences, University of Tampere, Tampere University Hospital, Tampere, Finland, [3] Department of Neurology, University of Tampere, Tampere University Hospital, Tampere, Finland, [4] Department of Surgical Sciences, Division of Anesthesiology and Intensive Care, Uppsala University, Uppsala, Sweden

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**Edited by:**
*J. Marc Simard, University of Maryland Baltimore, USA*

**Reviewed by:**
*Anke Höllig, Uniklinik RWTH Aachen, Germany*
*Caron Hong, University of Maryland Medical Center, USA*

***Correspondence:**
*Heikki Kiiski*
*heikki.kiiski@fimnet.fi*

†*These authors have contributed equally to this work.*

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**Object:** Aneurysmal subarachnoid hemorrhage (aSAH) is a common cause of death or long-term disability. Despite advances in neurocritical care, there is still only a very limited ability to monitor the development of secondary brain injury or to predict neurological outcome after aSAH. Soluble urokinase-type plasminogen activator receptor (suPAR) has shown potential as a prognostic and as an inflammatory biomarker in a wide range of critical illnesses since it displays an association with overall immune system activation. This is the first time that suPAR has been evaluated as a prognostic biomarker in aSAH.

**Methods:** In this prospective population-based study, plasma suPAR levels were measured in aSAH patients ($n$ = 47) for up to 5 days. suPAR was measured at 0, 12, and 24 h after patient admission to the intensive care unit (ICU) and daily thereafter until he/she was transferred from the ICU. The patients' neurological outcome was evaluated with the modified Rankin Scale (mRS) at 6 months after aSAH.

**Results:** suPAR levels ($n$ = 47) during the first 24 h after aSAH were comparable in groups with a favorable (mRS 0–2) or an unfavorable (mRS 3–6) outcome. suPAR levels during the first 24 h were not associated with the findings in the primary brain CT, with acute hydrocephalus, or with antimicrobial medication use during 5-days' follow-up. suPAR levels were associated with generally accepted inflammatory biomarkers (C-reactive protein, leukocyte count).

**Conclusion:** Plasma suPAR level was not associated with either neurological outcome or selected clinical conditions. While suPAR is a promising biomarker for prognostication in several conditions requiring intensive care, it did not reveal any value as a prognostic biomarker after aSAH.

**Keywords: aneurysmal subarachnoid hemorrhage, biomarkers, neurological outcome, secondary brain injury, soluble urokinase-type plasminogen activator receptor, neuroinflammation**

## INTRODUCTION

Urokinase plasminogen activator receptor (uPAR) (CD87) is present on various immunologically active cells, and its expression becomes elevated by inflammatory conditions and ischemic diseases (1, 2). The soluble form [soluble urokinase-type plasminogen activator receptor (suPAR)] in serum or plasma has emerged as an inflammatory biomarker capable of reflecting overall immune system activation (3, 4).

Previously, it has been shown that uPAR expression is induced in cerebral ischemia (5) and traumatic brain injury (6). Moreover, it has been claimed that uPAR may further augment cerebral injury (7). The induction of uPAR expression on the cell surface is believed to increase the levels of the soluble form of uPAR (8). Previously, suPAR has been shown to have predictive value in acutely, critically ill patients (9–13), including those who have suffered brain trauma (14). However, as far as we are aware, suPAR concentrations have not been evaluated as a prognostic biomarker in patients with subarachnoid hemorrhage.

Aneurysmal subarachnoid hemorrhage (aSAH) is a devastating disease causing long-term disability and up to 50% mortality (15). A significant proportion of the patients are young and previously healthy in comparison to individuals suffering other types of strokes (16). The main causes for poor prognosis are early brain injury and delayed cerebral ischemia (DCI), which cause permanent neurological deficits (17–19). The prediction of outcome is difficult and unsatisfactory as the secondary injury process in aSAH is multifactorial and incompletely understood (17, 20).

It is well established that inflammation plays a major role in vasospasm and subsequent DCI after aSAH (21, 22). A plethora of biomarkers have been studied in aSAH and DCI, e.g., neuron and astrocyte-specific markers (e.g., NSE, s100b, and UCHL-1), inflammatory biomarkers (e.g., IL-6, HMGB-1), and molecular adhesion and extracellular matrix markers (e.g., MMP-9) (16, 23–25). However, none of these putative biomarkers has so far proved to be useful in clinical decision-making. As ischemic events and inflammation are one characteristic feature of aSAH, it seemed reasonable to speculate that circulating plasma suPAR concentrations would increase during the acute stage after aSAH. Therefore, we hypothesized that either the plasma suPAR concentration or alternatively its changes over time could be useful in predicting the neurological outcome following aSAH.

## MATERIALS AND METHODS

The clinical data and blood samples from this patient cohort have been used in a previously published study (25). Following registration in Clinical Trials (NCT02026596, https://clinicaltrials.gov) and approval by the institutional ethics committee, we conducted a prospective, observational, single-center clinical study in Tampere University Hospital (Tampere, Finland) intensive care unit (ICU). The study population consisted of 61 consecutive adult aSAH patients admitted to our tertiary referral center during a 10-month period in 2013. Written informed consent was obtained from each of the patients or from their next of kin. All patients were treated according to standard in-house guidelines, which included intravenous nimodipine to prevent vasospasm and routine laboratory samples. In the final analyses, we chose to exclude those 14 patients with an unknown time of onset of symptoms or in whom the suPAR concentration was not measured during the first 24 h after the onset of symptoms. By including only those 47 patients with a known onset of clinical ictus and suPAR measurement during the first 24 h after the onset of symptoms, we eliminated the possibility that changes in suPAR concentrations would be attributable to different delays to hospital admission after aSAH.

The plasma suPAR concentration was measured at 0, 12, and 24 h after the admission and every 24 h for up to 5 days or until the patient was transferred from the ICU. World Federation of Neurological Surgeons Grading Scale, Fisher grade, and 6-month modified Rankin Scale (mRS) were used to evaluate the severity of aSAH and neurological recovery as previously described (25). The incidence of acute hydrocephalus was defined as the need for ventriculostomy on a clinical basis during the first 24 h after aSAH. Infection was defined as the need for antimicrobial medication during intensive care follow-up period.

As a part of our in-house guideline, an arterial cannula was routinely inserted. Blood samples for suPAR were collected into EDTA-containing tubes from the arterial cannula and the samples were immediately centrifuged for 10 min at 2,000 *g* at room temperature. After centrifugation, the plasma was collected and frozen at −70°C. After thawing, plasma suPAR levels were measured with a commercially available enzyme-linked immunosorbent assay kit according to the manufacturer's instructions (suPARnostic®, ViroGates, Birkeroed, Denmark). The detection limit and inter-assay coefficient of variation were 0.45 ng/ml and 3.2%, respectively.

Before the statistical analysis, the suPAR measurements were divided into consecutive 24 h intervals, starting from the onset of symptoms. If suPAR was measured more than once per interval, the mean concentration was used. In a subgroup of 22 patients who had up to 5-days' follow-up, we also checked whether the patient had been treated for DCI. Initiation of this treatment was based on clinical evaluation. Statistical analyses were performed with R (version 3.3.2 for Mac Os X). Fisher's exact test was used with the categorical variables. Due to the non-normal distribution of measured biomarkers, Mann–Whitney *U*-test was used for between-group comparisons. Correlations were evaluated with Spearman's correlation test. Linear regression was used in the time interval analyses.

## RESULTS

The basic characteristics of the study cohort and the subset of patients with 5-day follow-up have been previously reported (25). The time course of plasma suPAR concentrations in the two groups according to neurological outcome are depicted in **Figure 1**.

Plasma suPAR concentrations during the first 24 h after aSAH were comparable in the groups with a favorable (mRS 0–2) and an unfavorable (mRS 3–6) outcome (**Figure 2A**). Similarly, no differences were detected in the suPAR levels between those patients presenting with severe (WFNS 4–5) vs. non-severe (WFNS 1–3)

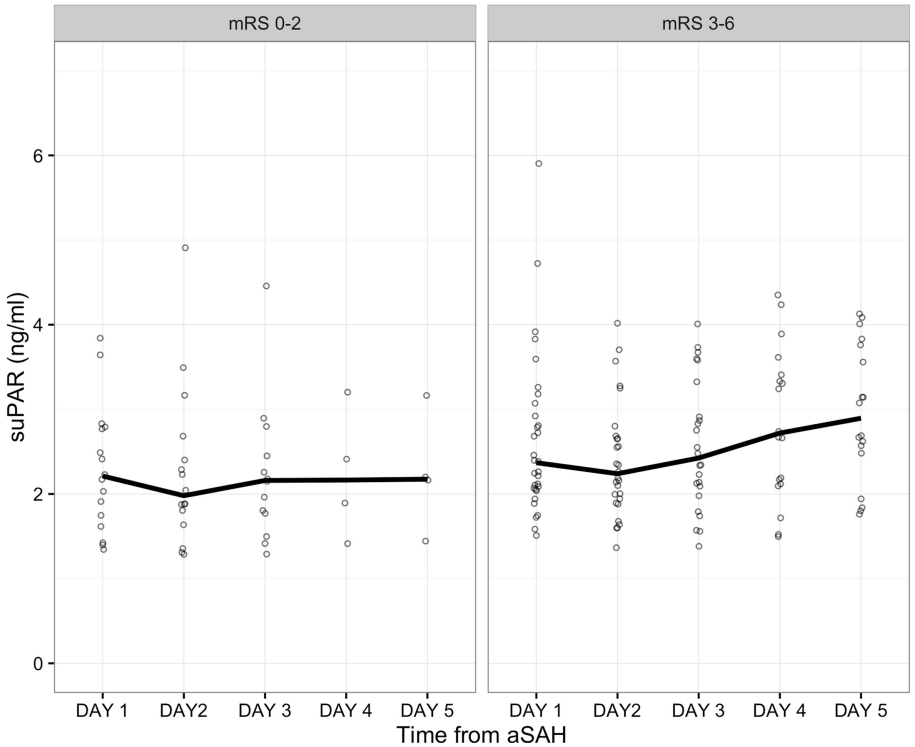


**FIGURE 1** | **Soluble urokinase-type plasminogen activator receptor (suPAR) levels in all patients and at all time intervals**. Values are grouped according to whether the patients had a favorable or an unfavorable neurological outcome. Dots represent individual patient values. The line represents group median.

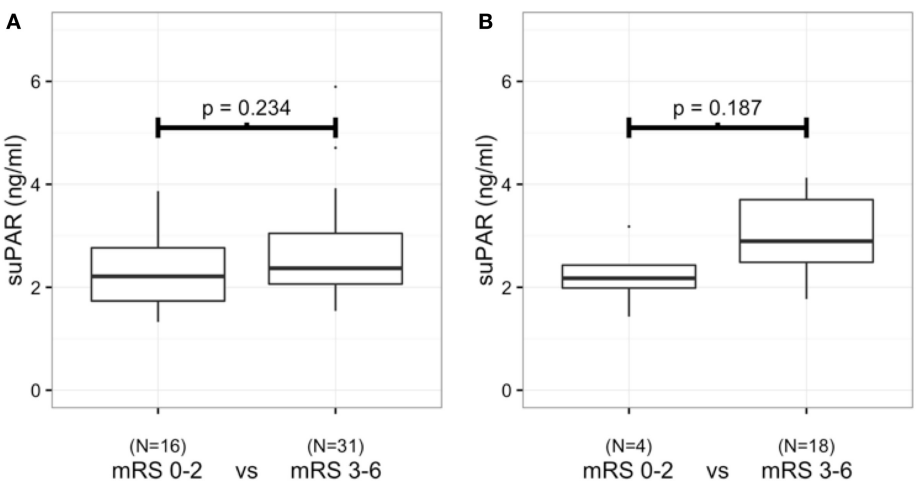


**FIGURE 2** | **Soluble urokinase-type plasminogen activator receptor (suPAR) levels during the first 24 h after aneurysmal subarachnoid hemorrhage between patients (*n* = 47) with a favorable or an unfavorable neurological outcome (A)**. suPAR levels at the end of 5-day follow-up between patients (*n* = 22) with a favorable or an unfavorable neurological outcome **(B)**.

findings in terms of their clinical status on admission (**Table 1**). Furthermore, the plasma suPAR concentration during the first 24 h was not associated with the findings from the primary brain CT (Fisher grade) with acute hydrocephalus or with antimicrobial medication during the 5-days' follow-up (**Table 1**). Age over 70 years was a strong predictor of an unfavorable neurological outcome, i.e., only one patient over 70 years experienced a neurologically favorable outcome ($p = 0.037$, **Table 2**).

suPAR was measured daily up to 5 days after the admission unless the patient died or was transferred from the ICU. In the 22 patients in whom we had suPAR concentrations measured up to 5 days, four patients achieved a favorable neurological outcome

**TABLE 1 | Soluble urokinase-type plasminogen activator receptor (suPAR) concentrations ($n = 47$) within 24 h from aneurysmal subarachnoid hemorrhage and the association with selected clinical conditions.**

| suPAR (ng/ml) | Mean | SD | Median | IQR | *p*-Value |
|---|---|---|---|---|---|
| Modified Rankin Scale | | | | | 0.234 |
| 0–2 ($n = 16$) | 2.30 | 0.75 | 2.21 | 1.73–2.77 | |
| 3–6 ($n = 31$) | 2.66 | 0.96 | 2.37 | 2.06–3.05 | |
| World Federation of Neurological Surgeons grading scale | | | | | 0.803 |
| 1–3 ($n = 28$) | 2.59 | 1.05 | 2.26 | 2.00–2.79 | |
| 4–5 ($n = 19$) | 2.47 | 0.66 | 2.41 | 2.00–2.86 | |
| Fisher | | | | | 0.240 |
| 1–2 ($n = 14$) | 2.36 | 0.93 | 2.05 | 1.67–2.78 | |
| 3–4 ($n = 33$) | 2.61 | 0.90 | 2.41 | 2.07–2.80 | |
| Infection | | | | | 0.402 |
| Yes ($n = 14$) | 2.55 | 0.64 | 2.61 | 2.21–2.88 | |
| No ($n = 33$) | 2.53 | 1.01 | 2.22 | 1.92–2.79 | |
| Acute hydrocephalus | | | | | 0.845 |
| Yes ($n = 19$) | 2.54 | 0.83 | 2.27 | 2.09–2.80 | |
| No ($n = 28$) | 2.53 | 0.97 | 2.34 | 1.92–2.83 | |

*Mann–Whitney U-test was used.*

**TABLE 2 | The relationship between neurological outcome and age.**

| | Age ≤ 70 | Age > 70 | *p*-Value |
|---|---|---|---|
| | | | 0.037 |
| Modified Rankin Scale (mRS) 0–2 | 15 | 1 | |
| mRS 3–6 | 20 | 11 | |

*Fisher's exact test was used.*

(mRS 0–2). The linear regression did not detect any statistically significant elevation of suPAR levels during the 5-days' follow-up in patients with either a favorable (mRS 0–2) or an unfavorable (mRS 3–6) neurological outcome (**Figure 3**). In addition, at day five, plasma suPAR concentrations were comparable in the two groups (**Figure 2B**).

Delayed cerebral ischemia treatment was initiated in 16/22 patients during the hospital stay in the subgroup in which we had at least a 5-day ICU follow-up. Neither DCI treatment nor infection or acute hydrocephalus was associated with plasma suPAR concentrations (**Table 3**).

The peak plasma suPAR concentration during the 5 days of follow-up was positively correlated with peak levels of C-reactive protein (CRP) ($p = 0.039$) and leukocyte numbers ($p = 0.006$). The plasma suPAR concentration was positively correlated with the leukocyte count during the first 24 h ($p = 0.049$). In contrast, suPAR levels were not associated with age (**Table 4**).

## DISCUSSION

The present study aimed to evaluate the potential prognostic value of plasma suPAR concentrations after an aSAH. In contrast to our working hypothesis, plasma suPAR did not show any association with neurological outcome, survival, acute hydrocephalus, clinical infection, or DCI in our patient cohort.

The inflammatory reaction and increase of systemic inflammatory mediators in aSAH is well documented (22, 26). Even though substantial evidence is accumulating in the literature highlighting the significant role of neuroinflammation in the outcome of aSAH, it is still unclear which, if any, inflammatory biomarkers can be used to guide clinical decision-making. The apparent biphasic nature of the inflammatory response after aSAH makes this challenge even more demanding. Neuroinflammation seems to have properties, which can be considered in some cases as protective, but in others, as deleterious, e.g., depending on the magnitude of the response, time-point after the ictus when activation of the inflammatory response occurs, and the type of cells recruited in the response (27). Hence, it is not surprising that there is inconsistency regarding the prognostic value of many inflammatory biomarkers such as IL-6 and HMGB1 after aSAH (19, 25, 28, 29). Nevertheless, although no biomarker has been identified, these studies have increased our understanding of the inflammatory process in aSAH and, in fact, also novel inflammatory biomarkers are claimed to have some prognostic potential, e.g., toll-like receptor 4 (30).

suPAR is considered as an inflammatory biomarker and mediator, a proposal that is well supported in the literature. Previously, increased serum or plasma suPAR levels have been postulated as a prognostic factor for poor outcome in critically ill patients with an inflammatory condition (31). The plasma suPAR concentrations in our aSAH cohort were low compared to septic and non-septic ICU patients with organ dysfunction (32). Nosocomial infections, organ dysfunction, and SIRS are frequent after aSAH (33, 34). Although the value of suPAR has been verified in infections and organ dysfunction (13, 32, 35), we did not detect high levels of suPAR, even later in the course of intensive care. One possible confounding factor distinguishing aSAH from other acute neurological conditions is that all of our patients received nimodipine to prevent DCI. Nimodipine has been shown to decrease plasminogen activator inhibitor 1 (PAI-1) activity (36). As PAI-1 is the major inhibitor of urokinase plasminogen activator (uPA), plasminogen activity and fibrinolysis may increase as a consequence of decreased PAI-1 activity. UPA can cleave the GPI-anchor on cell surface, but since a correct ratio of uPA is required for cleavage, it is possible that excess uPA due to nimodipine may inhibit the cleavage of suPAR from the cell surface (4, 8). suPAR also displays uPA dose dependence for binding to vitronectin (37), which may alter suPAR levels. Furthermore, previous reports have described suPAR-fragment release from activated neutrophils (38) and inhibition of neutrophil activation by two calcium antagonists, felodipine, and nimodipine (39), thereby supporting the concept that nimodipine may indeed be the factor modifying the suPAR response in our patient cohort. This speculative hypothesis and thus the potential direct effect of nimodipine on plasma suPAR concentrations could not be further tested/evaluated in our patient cohort. Moreover, as nimodipine is considered as part of current best practice, it would be unethical to establish a control group not receiving nimodipine after aSAH. Any further experiments to test this hypothesis will need to be conducted as preclinical/animal studies.

Overall, the suPAR response, i.e., the increase in the plasma concentrations of suPAR observed in this study, was rather modest in comparison with that observed in other critically ill patients, even though there was biochemically logical correlation

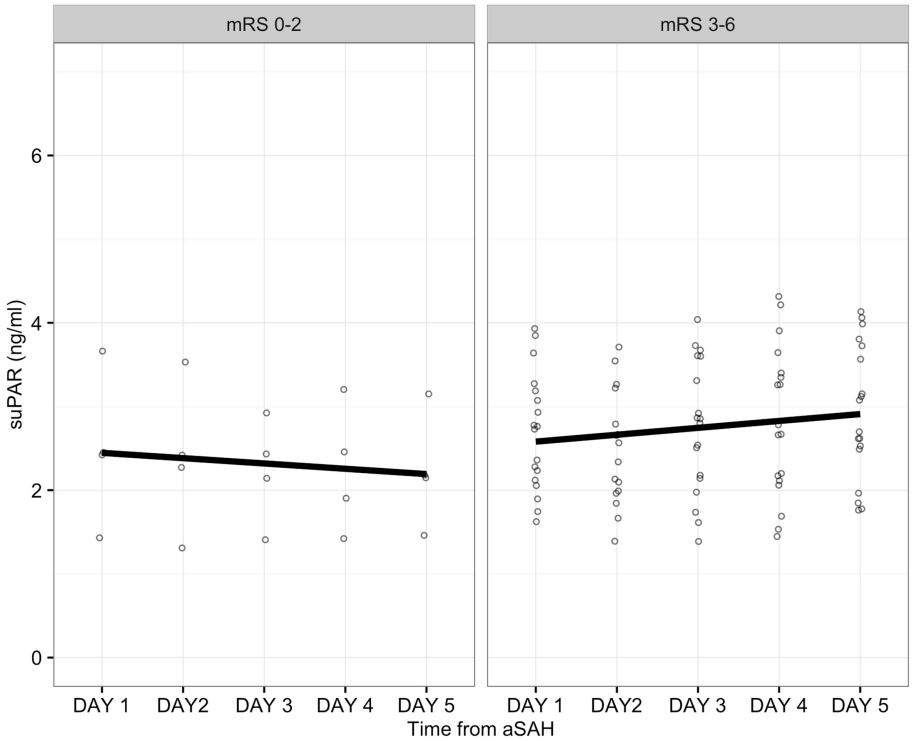


**FIGURE 3 | Soluble urokinase-type plasminogen activator receptor (suPAR) levels for patients with a follow-up of up to 5 days ($n = 22$).** Dots represent individual patient values. Regression line is calculated with linear regression. Values are grouped according to favorable ($p = 0.584$) or non-favorable ($p = 0.158$) neurological outcome.

**TABLE 3 | Soluble urokinase-type plasminogen activator receptor (suPAR) levels on day five from aneurysmal subarachnoid hemorrhage patients ($n = 22$) in whom there was 5-days' intensive care unit follow-up data and their association with selected clinical conditions.**

| suPAR (ng/ml) | Mean | SD | Median | IQR | *p*-Value |
|---|---|---|---|---|---|
| Modified Rankin Scale | | | | | 0.187 |
| 0–2 ($n = 4$) | 2.24 | 0.72 | 2.18 | 1.99–2.43 | |
| 3–6 ($n = 18$) | 2.95 | 0.81 | 2.90 | 2.49–3.70 | |
| Delayed cerebral ischemia treatment | | | | | 0.854 |
| Yes ($n = 16$) | 2.80 | 0.79 | 2.64 | 2.18–3.32 | |
| No ($n = 6$) | 2.89 | 1.00 | 3.12 | 2.25–3.48 | |
| Infection | | | | | 0.511 |
| Yes ($n = 11$) | 2.97 | 0.81 | 3.09 | 2.60–3.67 | |
| No ($n = 11$) | 2.68 | 0.86 | 2.46 | 2.01–3.17 | |
| Acute hydrocephalus | | | | | 0.511 |
| Yes ($n = 11$) | 2.70 | 0.91 | 2.64 | 1.91–3.45 | |
| No ($n = 11$) | 2.95 | 0.77 | 3.09 | 2.32–3.39 | |

*Mann–Whitney U-test was used.*

**TABLE 4 | Soluble urokinase-type plasminogen activator receptor and its association with leukocyte count, C-reactive protein (CRP), and age.**

| | Spearman rho | *p*-Value |
|---|---|---|
| **Day 1 ($n = 47$)** | | |
| Leukocyte count | 0.289 | 0.049 |
| CRP | 0.261 | 0.077 |
| AGE | 0.228 | 0.123 |
| **5-day intensive care unit follow-up ($n = 22$)** | | |
| Maximum leukocyte count during follow-up | 0.568 | 0.006 |
| Maximum CRP during follow-up | 0.443 | 0.039 |
| Age | 0.143 | 0.527 |

between suPAR levels and generally accepted inflammatory biomarkers (CRP, leukocyte count). The correlation, however, was weak in comparison to previously reported results (35, 40) possibly indicating that there are numerous factors influencing inflammatory biomarkers and mediators in aSAH. In addition, although higher age was associated with poor outcome *per se*, in our patient cohort, we observed no correlation between age and plasma suPAR levels. This finding contradicts the results of several previous studies (10, 13, 41, 42). Finally, the low incidence (29.8%) of nosocomial infections in our patient cohort may partially explain the observed low plasma suPAR levels.

Even though serum suPAR levels have been shown to be elevated in ischemic stroke (43) and in cerebrospinal fluid (CSF) following disruption of the blood–brain barrier (44), no marked elevation of plasma suPAR was found in patients either diagnosed with DCI or acute hydrocephalus. Further analyses will be necessary to clarify potential importance of suPAR release to CSF in patients with DCI and acute hydrocephalus. In order to reveal the actual role of suPAR as a biomarker or mediator in aSAH, it would be worthwhile evaluating the potential value of suPAR levels in CSF in diagnosing ventriculitis related to ventriculostomy catheter.

Our study has some limitations. First, we had some patients that were lost to follow-up. In other words, we were not able to obtain samples late in the course of the acute illness as patients had either died or been transferred to some other health-care facility. These patients represent two extremes, i.e., either the best or the worst outcome, and this may have altered the results of our analysis. Second, our sample size was limited. In particular, only four patients with a favorable neurological outcome remained in the final analyses on day 5. However, some of our patients experienced mild whereas others had very severe presentations of aSAH. We followed suPAR levels during the whole ICU stay and suPAR levels were constantly low with no high peaks being observed. This suggests that aSAH does not induce high suPAR levels in plasma or they are depressed by some aspect of the treatment, for example, administration of the calcium antagonist. Third, our study is a single-center study. Although our unit is a tertiary referral hospital with a high patient influx, single-center bias is possible. Despite their relatively low numbers, it is of interest that those patients with a good neurological outcome had remarkably low plasma suPAR levels with a very small SD (**Figure 2B**). Our previous studies have suggested that while high suPAR levels may be prognostic for poor outcome, in contrast, a low plasma suPAR concentration is predictive of a good outcome (11, 45). In the present study, the number of patients is limited, but the same phenomenon may apply to aSAH.

## CONCLUSION

This study reports the first population-based prospective, observational results evaluating plasma suPAR concentrations in aSAH. Plasma suPAR levels were not associated with neurological outcome or selected clinical conditions. While suPAR is a promising biomarker in several conditions requiring intensive care, based on this study, it does not seem to be useful as a prognostic biomarker in patients with aSAH.

## ETHICS STATEMENT

The study was approved by the Ethics Committee of Pirkanmaa Hospital District. Written informed consent was obtained from each of the patients or from the next of kin.

## AUTHOR CONTRIBUTIONS

HK contributed to study conception, neurological outcome evaluations, statistical analyses, and drafted of the manuscript. VJ contributed to patient recruitment, statistical analyses, and drafted the manuscript. MA-P contributed to patient recruitment, evaluation of the initial clinical severity, evaluation of the initial computed tomography findings, and drafted the manuscript. MH contributed to the laboratory analysis of suPAR and drafted the manuscript. EM contributed to the laboratory analysis of suPAR and drafted the manuscript. JP contributed to neurological outcome evaluations, and drafted the manuscript. JT contributed to study conception, statistical analyses, and drafted the manuscript.

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# High Compliance with Scheduled Nimodipine Is Associated with Better Outcome in Aneurysmal Subarachnoid Hemorrhage Patients Cotreated with Heparin Infusion

*Aaron Wessell[1], Matthew J. Kole[1], Neeraj Badjatia[1,2,3], Gunjan Parikh[2], Jennifer S. Albrecht[4], David L. Schreibman[1,3] and J. Marc Simard[1,5,6]**

[1] Department of Neurosurgery, University of Maryland School of Medicine, Baltimore, MD, United States, [2] Department of Neurology, University of Maryland School of Medicine, Baltimore, MD, United States, [3] Department of Anesthesiology, University of Maryland School of Medicine, Baltimore, MD, United States, [4] Department of Epidemiology and Public Health, University of Maryland School of Medicine, Baltimore, MD, United States, [5] Department of Pathology, University of Maryland School of Medicine, Baltimore, MD, United States, [6] Department of Physiology, University of Maryland School of Medicine, Baltimore, MD, United States

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**Edited by:**
Barak Bar,
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Thomas Jefferson University Hospital, United States

***Correspondence:**
J. Marc Simard
msimard@som.umaryland.edu

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**Introduction:** We sought to determine whether compliance with scheduled nimodipine in subarachnoid hemorrhage patients impacted patient outcomes, with the intent of guiding future nimodipine management in patients who experience nimodipine-induced hypotension.

**Methods:** We performed a retrospective analysis of 118 consecutive aneurysmal subarachnoid hemorrhage patients treated with the Maryland Low-Dose IV Heparin Infusion Protocol. Patients were categorized into three independent nimodipine compliance groups: ≥1 dose held, ≥1 dose split, and no missed or split-doses. A split-dose was defined as 30 mg of nimodipine administered every 2 h. Our primary outcome was discharge to home. Bivariate and multivariable logistic regression analyses were used to assess predictors of discharge disposition as a function of nimodipine compliance.

**Results:** Of the 118 patients, 20 (17%) received all nimodipine doses, 6 (5%) received split-doses but never had a full dose held, and 92 (78%) had ≥1 dose held. Forty-five percent of patients were discharged to home, including 75% who received all doses, 67% who received ≥1 split-doses, and 37% with ≥1 missed doses ($p = 0.003$). Multivariable analysis showed that, along with age and World Federation of Neurosurgical Societies grade, nimodipine compliance was an independent predictor of clinical outcome; compared to missing one or more nimodipine doses, full dosing compliance was associated with increased odds of discharge to home (odds ratio 5.20; 95% confidence intervals 1.46–18.56).

**Conclusion:** In aneurysmal subarachnoid hemorrhage patients with modified Fisher scores 2 through 4 who are cotreated with a low-dose heparin infusion, full compliance with nimodipine dosing was associated with increased odds of discharge to home.

**Keywords: nimodipine, subarachnoid hemorrhage, cerebral aneurysm, cerebral vasospasm, heparin, vascular disorders**

## INTRODUCTION

Aneurysmal subarachnoid hemorrhage is associated with substantial morbidity and mortality (1). Approximately one-third of those who survive the initial hemorrhage remain dependent, while another 20–30% who present with a low clinical grade will experience further decline from their admission neurologic status (1, 2). For those who survive the ictus, the onset of delayed neurological deficit (DND), occurring in nearly 30% of patients, is the most important factor impacting patient outcome (3, 4). DND refers collectively to the neurological, psychological, and cognitive effects experienced by those who have suffered a subarachnoid hemorrhage. The mechanisms implicated in DND include arterial vasospasm, microthrombosis, oxidative damage, and neuroinflammation, with resultant cerebral infarction, demyelination, cerebral edema, endothelial, and neuronal cell death (5–14).

Nimodipine is the only pharmacological agent that has been consistently shown in randomized, placebo-controlled trials to improve outcomes in aneurysmal subarachnoid hemorrhage (15–19). Nimodipine is a lipophilic dihydropyridine calcium-channel blocker that crosses the blood–brain barrier and prevents the influx of extracellular calcium into vascular smooth muscle, neuronal, and other cells (20). Nimodipine may exhibit a degree of selectivity for the cerebral vasculature due to its greater dependence on extracellular calcium for smooth muscle contraction. However, hypotension, along with other adverse systemic effects such as edema, abnormal liver function, headache, flushing, and gastrointestinal symptoms, have been reported with nimodipine use (15–19).

A significant percentage of patients admitted with aneurysmal subarachnoid hemorrhage demonstrate sensitivity to nimodipine's hypotensive effect. This presents a clinical dilemma that served as the impetus for the present study—should nimodipine dosing be reduced or stopped in the face of drug-induced hypotension, or should dosing be maintained, with vasopressors used as required to counteract hypotension? Patients who cannot tolerate nimodipine will not benefit from its neuroprotectant, anti-inflammatory, and pro-fibrinolytic effects (21–33), and consequently may have poorer outcomes after subarachnoid hemorrhage.

The purpose of this study was to assess the association between nimodipine dosing compliance and clinical outcome at time of discharge after aneurysmal subarachnoid hemorrhage in patients cotreated with a continuous low-dose heparin infusion.

## MATERIALS AND METHODS

### Patient Selection

The present study was conducted with approval of the University of Maryland Medical Center IRB. The records of all patients treated for aneurysmal subarachnoid hemorrhage by a single cerebrovascular neurosurgeon (J. Marc Simard) between July 1, 2008 and July 1, 2015 were reviewed. Patients underwent surgical clipping, endovascular coiling, or a combination of the two. All patients underwent treatment with the Maryland Low-Dose IV Heparin Infusion Protocol, consisting of 12 U/kg/h of unfractionated heparin, administered by constant IV infusion, beginning 12 h after aneurysm treatment, which is a practice pattern of the senior author (34–36). Patients with modified Fisher scores of 2 through 4 were included, as this group is at greatest risk of developing cerebral vasospasm. Those with insufficient hospital and medication-administration records were excluded from analysis. None of the patients in the present cohort were included in a previous retrospective analysis by Simard et al. published in 2013 (36).

### Management of Aneurysmal Subarachnoid Hemorrhage Patients

All patients were treated according to the American Heart Association's Guidelines for the Management of Aneurysmal Subarachnoid Hemorrhage (37, 38), with an emphasis on prompt aneurysm obliteration, drainage of cerebrospinal fluid for treatment of hydrocephalus, maintenance of euvolemia, and administration of oral nimodipine. Radiographic studies, such as computed tomography (CT), CT angiogram (CTA), digital subtraction angiogram (DSA), and/or magnetic resonance angiography, were obtained routinely within 24 h of treatment to confirm obliteration of the aneurysm, rule out any complication of treatment, and evaluate for the development of hydrocephalus.

If patients experienced neurological deterioration attributed to vasospasm, regardless of radiographic confirmation, patients were treated with hemodynamic augmentation consisting of IV fluid bolus to assure euvolemia and vasopressor-induced hypertensive therapy with phenylephrine or norepinephrine to increase systolic blood pressure by 20–40%. The decision to obtain vascular imaging for evaluation of vasospasm, such as a CTA or DSA, was at the discretion of the attending neurointensivist. Daily monitoring for vasospasm with transcranial Doppler or electroencephalogram was not routinely performed. If clinical deterioration persisted despite hemodynamic augmentation, and radiographic study confirmed the presence of vasospasm, selective catheter angiography was performed with administration of intra-arterial calcium-channel blocker. Angioplasty was performed at the discretion of the attending neuro-interventional radiologist in select cases when intra-arterial vasodilator therapy did not produce a sufficient radiographic improvement in blood vessel caliber and/or arterial filling as observed on catheter cerebral angiogram.

### Nimodipine Use in Subarachnoid Hemorrhage

Patients were treated with 60 mg of oral nimodipine every 4 h beginning within 96 h of the ictus according to the AHA guidelines (37). At our institution, patients are initiated on oral nimodipine at the time of admission and are maintained on this medication until discharge, up to a maximum of 21 days. The onset of hypotension (systolic blood pressure <90 mm Hg) following administration of nimodipine, regardless of whether or not a patient was actively undergoing treatment with vasopressor-induced hypertension, may have prompted a change in dosing to 30 mg every 2 h. Alternatively, a given dose may have been withheld if the hypotension was lasting, associated with a change

in neurological examination, or associated with reoccurring blood pressure drops in the setting of active vasospasm requiring frequent rescue therapy with vasopressors. The decision to do so was based on individual patient characteristics, as determined by the attending neurointensivist.

## The Maryland Low-Dose IV Heparin Infusion Protocol

The Maryland Low-Dose IV Heparin Infusion Protocol for use in aneurysmal subarachnoid hemorrhage has been described previously (36). In the original report, use of a low-dose heparin infusion for approximately 14 days after aneurysm treatment was associated with a reduced incidence of DND, requirement for rescue therapy, cerebral infarction, and discharge to inpatient rehabilitation (36). Heparin is a pleiotropic drug that may antagonize several biomolecular pathways implicated in delayed neurologic decline following aneurysmal subarachnoid hemorrhage. Unfractionated heparin complexes with oxyhemoglobin, blocks the activity of free radicals, antagonizes endothelin-mediated vasoconstriction, smooth muscle depolarization, and inflammation (34, 35). All of the patients included in the present analysis underwent treatment with a low-dose heparin infusion. On post-bleed day 14, a screening CTA with perfusion analysis was performed to assess for radiographic vasospasm. If there was only mild or no vasospasm, the heparin infusion was discontinued and in the patient was considered for downgrading to a lower level of care if they did not have a ventriculostomy catheter in place. In select cases of low World Federation of Neurosurgical Societies (WFNS) grade subarachnoid hemorrhage, if the patient had not suffered any delayed neurologic decline and had a stable neurologic examination, an early screening CTA (at post-bleed day 10, for example) was performed and the patient was then downgraded to a lower level of care as deemed appropriate by the ICU provider.

## Data Compilation

Patient records, including hospital records, operative reports, clinic notes, and radiographic studies, were reviewed. Demographic, clinical, radiographic, and outcomes data were compiled.

The occurrence of angiographic vasospasm was determined from review of CTA and DSA studies by two neurosurgeons and independently by neuroradiologists from the University of Maryland. Studies demonstrating angiographic vasospasm in any vascular territory in comparison to a baseline study were characterized as mild (0–33%), moderate (34–66%), or severe (67–100%) vessel narrowing, independent of the patient's clinical/neurologic status (39). The incidence of DND was determined from review of the patient's neurologic status as documented in hospital records. The presence of angiographic vasospasm in a patient with neurological deterioration was deemed clinical vasospasm, or a delayed neurologic deficit. DND was further defined to include any neurologic decline, such as a change in mental status, pronator drift, or focal neurologic deficit without any other attributable medical causes regardless of radiographic confirmation of vasospasm.

The occurrence of new ischemia-related CT hypodensities was determined from review of all CT scans by two neurosurgeons and independently by neuroradiologists from the University of Maryland. CT scans obtained during hospitalization and on follow-up were correlated with CTA or DSA and neurological examination to determine whether new hypodensities were attributable to vasospasm (within a discrete vascular or watershed territory), as opposed to resulting from intraparenchymal hemorrhage, intraventricular catheter placement, surgical retraction, or surgery-related loss of a perforating artery.

## Exposure Definition

We created three independent groups based on compliance with nimodipine dosing during hospitalization. Nimodipine administration was evaluated by review of medication-administration records. Blood pressure data were obtained from review of nursing flow sheets recorded during the first 21 days of hospitalization following aneurysm rupture. Nimodipine doses held for relative hypotension, irrespective of whether or not a patient was actively undergoing treatment with vasopressor-induced hypertension, were recorded. Nimodipine administered as 30 mg every 2 h was considered a split-dose. A patient who required a split-dose but later had a dose held was grouped among patients who missed one or more complete doses. Patients receiving all scheduled doses, with no split-doses received, were the final group. A small number of patients ($n = 5$, 4%) never received Nimodipine due to significant hemodynamic instability on admission or cardiac dysfunction and were grouped with the doses held group. Dosing alterations during surgical or endovascular treatment were not considered in the analysis.

## Outcome Definition

We defined a good outcome as discharge to home. Discharge disposition previously has been reported as an outcome measure in studies of subarachnoid hemorrhage, serving as a surrogate for short-term functional outcome (40, 41). While several non-clinical factors may influence one's discharge disposition, past reports have shown that discharge status correlates with modified Rankin Scale score and functional outcome at 90 days in stroke patients (40, 42). Recommendations for discharge to home versus inpatient rehabilitation or nursing facility were made independently by physical and/or occupational therapists according to activities of daily living based on the patient's degree of functional independence. All other discharge locations, including in-hospital mortality, comprised "discharge to other." Glasgow Outcomes Scale (GOS) scores at time of discharge were calculated in a retrospective manner from clinical documentation.

We assessed the distribution and frequency of all variables. Covariates were compared between nimodipine compliance categories using the Fisher–Freeman–Halton test, ANOVA, or the Kruskal–Wallis test. Covariates were compared between outcome groups using Chi-Square Goodness of Fit, Student's *t*-test, or the Wilcoxon rank sum test. We assessed the unadjusted association between nimodipine compliance categories and discharge to home using logistic regression. Covariates that were associated with the exposure and outcome but not in the causal pathway (i.e., did not occur after initial nimodipine exposure) were considered for inclusion in our final logistic regression model (43, 44). We

assessed potential effect modification *a priori* by age and WFNS grade by creating interaction terms in the logistic regression model. Odds ratios (OR) and 95% confidence intervals (CI) are reported.

Due to the small number of patients who received a split-dose of nimodipine but did not miss any doses, we conducted sensitivity analyses to assess whether inclusion of these individuals with the other compliance groups would change effect estimates. All analyses were conducted using SAS version 9.3 (SAS Institute, Cary, NC, USA). A $p$-value <0.05 was considered significant.

# RESULTS

## Demographic Characteristics

Data from 118 consecutive patients were analyzed, all of whom were treated with the Maryland Low-Dose IV Heparin Infusion Protocol (36). The demographic characteristics of patients, grouped according to their nimodipine compliance, are shown in **Table 1**. Age, sex, medical history, WFNS grade, GCS motor score, and modified Fisher score did not differ significantly among compliance groups. Anterior cerebral artery aneurysms were the most common (45.8%). The majority of patients underwent treatment of their aneurysm within 20 h of admission, with 108 patients undergoing surgical clipping and 10 undergoing endovascular coiling. 11 (10.2%) patients had an intraparenchymal hematoma evacuated during open surgery. Admission echocardiograms were performed on 45 (38%) patients with a mean ejection fraction of 60.87% (SD, 13.63).

## Nimodipine Use

Twenty patients (17%) received 100% of the scheduled nimodipine doses from the time of admission through the end of their treatment period, 6 patients (5%) received at least one split-dose, and 92 (78%) missed one or more doses.

## Vasospasm

A total of 79 patients (67%) were found to have angiographic vasospasm on CTA or DSA, with 27 (23%) of those cases being severe. As shown in **Table 1**, vasospasm severity ($p = 0.04$), DND ($p = 0.03$), use of vasopressor-induced hypertension ($p = 0.01$), days on vasopressors ($p = 0.008$), and the use of intra-arterial therapy ($p = 0.003$) differed significantly among compliance groups. There was no significant difference in the use of angioplasty or systolic blood pressure.

## Outcomes

Overall, patients suffered a very low rate of CT infarction, with an incidence of 12%. As shown in **Table 1**, the incidence of CT infarction, shunt insertion, and death did not differ significantly between compliance groups. Patients with full compliance were more likely to be discharged home (75%), compared to those who received one or more split nimodipine doses (67%) or those who missed one or more full-doses (37%) ($p = 0.003$). Similarly, patients with full compliance tended to have a shorter ICU stay ($p = 0.04$) and GOS > 3 at discharge (95%) compared to those who received one or more split-doses (83%) or those who missed one or more full-doses (71%) ($p = 0.05$). All patients who died missed one or more nimodipine doses.

## Discharge Disposition

The characteristics of patients grouped by discharge disposition are shown in **Table 2**. Fifty-three patients (45%) were discharged to home and 65 (55%) were discharged to other locations, including 7 who died. There were no readmissions for late DND after discharge. In bivariate analysis, age ($p < 0.001$), WFNS grade ($p < 0.001$), admission GCS motor score ($p = 0.003$), and modified Fisher score ($p = 0.002$) were significantly associated with discharge disposition, whereas the only medical history that was significantly associated was hypertension ($p = 0.03$).

Nimodipine dosing compliance was significantly associated with discharge disposition ($p = 0.003$). Also, vasopressor-induced hypertension, systolic blood pressure, days on vasopressors, and the use of intra-arterial therapy were significantly associated with discharge disposition (all $p < 0.001$). In addition, DND, CT infarction, shunt insertion, and ICU length of stay were significantly associated with discharge disposition ($p$ values ranging from 0.04 to <0.001).

Prior to adjustment for confounding, we found that full nimodipine dosing compliance was significantly associated with discharge to home (OR, 5.12; 95% CI, 1.71–15.33) compared to missing at least one dose. Receiving at least one split-dose but not missing any doses also was associated with discharge to home (OR, 3.41; 95% CI, 0.59–19.62), but this association was not statistically significant.

Our final logistic regression model included an indicator for the nimodipine compliance categories, age, and a WFNS grade indicator (3 and 4 were grouped due to small cell sizes) (**Table 3**). There was no effect modification by age or WFNS grade. Full nimodipine dosing compliance was significantly associated with discharge to home (OR, 5.20; 95% CI, 1.46–18.56). In addition, greater age (OR, 0.95; 95% CI, 0.91–0.98), and WFNS grades 3/4 (OR, 0.16; 95% CI, 0.05–0.60) or 5 (OR, 0.12; 95% CI, 0.02–0.70) were significantly associated with poor discharge disposition. Receiving a split-dose of nimodipine (30 mg every 2 h) was not significantly associated with a worse outcome. Sensitivity analyses revealed that varying groupings by receiving a split-dose of nimodipine had no impact on effect estimates (**Table 4**).

# DISCUSSION

Nimodipine is the only pharmacologic agent shown in randomized placebo-controlled trials to improve outcome (15–19). Despite its widespread use, there are little data to guide the management of hypotension following nimodipine administration. The original randomized-controlled trial of nimodipine by Allen et al. (15) did not report any hypotensive effects. Subsequent studies comprised of patients with higher clinical grades reported various adverse effects of nimodipine, most commonly hypotension, leading to early cessation of the medication (16–19). In our study population, 45% of patients had a WFNS grade of III or higher, and 78% of patients experienced hypotension requiring that one or more doses be held.

**TABLE 1** | Characteristics of patients with subarachnoid hemorrhage from brain aneurysm rupture by nimodipine compliance, *n* = 118.

| Characteristic | Full nimodipine compliance, *n* = 20 | At least one nimodipine dose split, *n* = 6 | At least one nimodipine dose held, *n* = 92 | *p*-Value[a] |
|---|---|---|---|---|
| Age, mean (SD) | 51.4 (10.3) | 58.3 (9.9) | 56.5 (13.0) | 0.23 |
| Female, *n* (%) | 14 (70) | 4 (67) | 59 (64) | 0.93 |
| History of tobacco use, *n* (%) | 11 (55) | 2 (33) | 51 (55) | 0.64 |
| History of hypertension, *n* (%) | 12 (60) | 4 (67) | 57 (62) | >0.99 |
| History of statin use, *n* (%) | 2 (10) | 0 | 6 (7) | 0.76 |
| History of cocaine use, *n* (%) | 0 | 0 | 3 (3) | >0.99 |
| History of marijuana use, *n* (%) | 2 (10) | 0 | 6 (7) | 0.76 |
| WFNS (admission), *n* (%) | | | | 0.17 |
| 1 | 8 (40) | 4 (67) | 19 (21) | |
| 2 | 4 (20) | 0 | 30 (33) | |
| 3 | 0 | 0 | 1 (1) | |
| 4 | 7 (35) | 1 (17) | 33 (36) | |
| 5 | 1 (5) | 1 (17) | 9 (10) | |
| GCS motor score, *n* (%) | | | | 0.74 |
| 6 | 16 (80) | 4 (67) | 61 (66) | |
| 5 | 3 (15) | 1 (17) | 21 (23) | |
| 4 | 1 (5) | 0 | 5 (5) | |
| <4 | 0 | 1 (17) | 5 (5) | |
| MFS (admission), *n* (%) | | | | 0.39 |
| 2 | 3 (15) | 1 (17) | 5 (5) | |
| 3 | 14 (70) | 4 (67) | 70 (76) | |
| 4 | 3 (15) | 1 (17) | 17 (18) | |
| Aneurysm location, *n* (%)[b] | | | | 0.69 |
| Internal carotid artery | 6 (30) | 2 (33) | 23 (26) | |
| Anterior cerebral artery | 6 (30) | 3 (50) | 45 (49) | |
| Middle cerebral artery | 7 (35) | 1 (17) | 18 (20) | |
| Posterior circulation | 1 (5) | 0 | 6 (7) | |
| Procedure type, *n* (%) | | | | 0.82 |
| Clip | 19 (95) | 6 (100) | 83 (90) | |
| Coil | 1 (5) | 0 | 9 (10) | |
| >20 h to surgery, *n* (%) | 7 (35) | 2 (33) | 35 (38) | >99.0 |
| Vasospasm severity, *n* (%) | | | | 0.04 |
| none | 11 (55) | 3 (50) | 25 (27) | |
| mild | 0 | 2 (33) | 20 (22) | |
| moderate | 5 (25) | 0 | 25 (27) | |
| severe | 4 (20) | 1 (17) | 22 (24) | |
| VIH, *n* (%) | 3 (15) | 3 (50) | 46 (50) | 0.01 |
| Systolic blood pressure, mean (SD) | 139.2 (15.5) | 148.1 (16.4) | 145.2 (20.5) | 0.41 |
| Days on pressors, mean (SD) | 0.3 (0.8) | 5.2 (6.5) | 2.9 (4.2) | 0.008 |
| IAT needed, *n* (%) | 0 | 1 (17) | 22 (24) | 0.03 |
| Angioplasty, *n* (%) | 0 | 0 | 6 (7) | 0.70 |
| DND, *n* (%) | 4 (20) | 3 (50) | 48 (52) | 0.03 |
| CT infarction, *n* (%) | 1 (5) | 1 (17) | 12 (13) | 0.45 |
| Shunt inserted, *n* (%) | 2 (10) | 2 (33) | 27 (29) | 0.17 |
| ICU length of stay, mean (SD) | 15.7 (7.0) | 25.3 (18.5) | 19.9 (8.5) | 0.04 |
| GOS > 3, *n* (%) | 19 (95) | 5 (83) | 65 (71) | 0.05 |
| Discharged home, *n* (%) | 15 (75) | 4 (67) | 34 (37) | 0.003 |
| Died, *n* (%) | 0 | 0 | 7 (8) | 0.55 |

[a]*p-Values from the Fisher–Freeman–Halton test, ANOVA, or the Kruskal–Wallis test.*

*WFNS, World Federation of Neurosurgical Societies Scale; GCS, Glasgow Coma Scale; MFS, Modified Fisher Scale; DND, delayed neurological deficit; VIH, vassopressor-induced hypertension; IAT, intra-arterial treatment; ICU, intensive care unit; GOS, Glasgow Outcomes Scale.*

[b]*Aneurysms by location: anterior cerebral artery (A1, anterior communicating artery, A2 and pericallosal aneurysms); middle cerebral artery; internal carotid artery (anterior choroidal artery, posterior communicating artery, and carotid terminus aneurysms); posterior circulation (vertebral artery, basilar artery, posterior inferior cerebellar artery, anterior inferior cerebellar artery and superior cerebellar artery aneurysms).*

The Neurocritical Care Society's Multidisciplinary Consensus Conference states that if nimodipine causes hypotension, then dosing should be lowered and administered at more frequent intervals (38). Alternative dosing options include splitting the nimodipine dose into a 30 mg dose given every 2 h, halving the nimodipine dose by giving 30 mg every 4 h, holding nimodipine altogether, or administering volume resuscitation or vasopressor agents to counteract the hypotensive effects. Our institutional practice is that, if patients develop hypotension, dosing of nimodipine is changed to 30 mg every 2 h, and if patients continue to experience hypotension, nimodipine may be held altogether.

**TABLE 2** | Characteristics of patients with subarachnoid hemorrhage from brain aneurysm rupture 2008–2015 by discharge to home, *n* = 118.

| Characteristic | Discharge home, *n* = 53 | Discharge to Other location, *n* = 65 | *p*-Value[a] |
|---|---|---|---|
| Age, mean (SD) | 51.3 (11.1) | 59.4 (12.4) | <0.001 |
| Female, *n* (%) | 34 (64.2) | 43 (66.2) | 0.82 |
| History of tobacco use, *n* (%) | 25 (47.2) | 39 (60.0) | 0.16 |
| History of hypertension, *n* (%) | 27 (50.9) | 46 (70.8) | 0.03 |
| History of statin use, *n* (%) | 3 (5.7) | 5 (7.7) | 0.66 |
| History of cocaine use, *n* (%) | 1 (1.9) | 2 (3.1) | >0.99 |
| History of marijuana use, *n* (%) | 4 (7.6) | 4 (6.2) | >0.99 |
| WFNS, *n* (%) | | | <0.001 |
| 1 | 21 (39.6) | 10 (15.4) | |
| 2 | 20 (37.7) | 14 (21.5) | |
| 3 | 0 | 1 (1.5) | |
| 4 | 10 (18.9) | 31 (47.7) | |
| 5 | 2 (3.8) | 9 (13.9) | |
| GCS motor score, *n* (%) | | | 0.003 |
| 6 | 46 (87) | 35 (54) | |
| 5 | 5 (9) | 20 (31) | |
| 4 | 2 (4) | 4 (6) | |
| <4 | 0 | 6 (9) | |
| MFS, *n* (%) | | | 0.002 |
| 2 | 5 (9.4) | 4 (6.2) | |
| 3 | 46 (86.8) | 42 (64.6) | |
| 4 | 2 (3.8) | 19 (29.2) | |
| Aneurysm location, *n* (%)[b] | | | 0.39 |
| Internal carotid artery | 12 (23) | 19 (29) | |
| Anterior cerebral artery | 22 (42) | 32 (49) | |
| Middle cerebral artery | 15 (28) | 11 (17) | |
| Posterior circulation | 4 (8) | 3 (5) | |
| Procedure type, *n* (%) | | | 0.18 |
| Clip | 51 (96.2) | 57 (87.7) | |
| Coil | 2 (3.8) | 8 (12.3) | |
| >20 h to surgery, *n* (%) | 18 (34.0) | 26 (40.0) | 0.50 |
| Nimodipine compliance, *n* (%) | | 5 (8) | 0.18 |
| All doses received | 15 (28) | 5 (8) | |
| At least one dose split | 4 (8) | 2 (3) | |
| At least one dose missed | 34 (64) | 58 (89) | |
| Vasospasm severity, *n* (%) | | | 0.05 |
| none | 21 (39.6) | 18 (27.9) | |
| mild | 12 (22.6) | 10 (15.4) | |
| moderate | 14 (26.4) | 16 (24.6) | |
| severe | 6 (11.3) | 21 (32.3) | |
| VIH, *n* (%) | 12 (22.6) | 40 (61.5) | <0.001 |
| Systolic blood pressure, mean (SD) | 134.5 (20.0) | 152.4 (15.3) | <0.001 |
| Days on pressors, M (IQR) | 0 (0,0) | 2.0 (0, 6.0) | <0.001 |
| IAT needed, *n* (%) | 3 (5.7) | 20 (30.8) | <0.001 |
| Angioplasty, *n* (%) | 0 | 6 (9.2) | 0.02 |
| DND, *n* (%) | 12 (22.6) | 43 (66.2) | <0.001 |
| CTinfarction, *n* (%) | 1 (1.9) | 13 (20.0) | 0.003 |
| Shunt inserted, *n* (%) | 9 (17.0) | 22 (33.9) | 0.04 |
| ICU length of stay, mean (SD) | 15.5 (5.4) | 22.8 (10.2) | <0.001 |
| GOS > 3, *n* (%) | 53 (100) | 36 (55.4) | <0.001 |
| Died, *n* (%) | 0 | 7 (10.8) | 0.01 |

[a]*p-Value from Chi-square Goodness of Fit, Student's t-test, or Wilcoxon rank sum.*

*WFNS, World Federation of Neurosurgical Societies Scale; GCS, Glasgow Coma Scale; MFS, Modified Fisher Scale; DND, delayed neurological deficit; VIH, vassopressor-induced hypertension; IAT, intra-arterial treatment; ICU, intensive care unit; GOS, Glasgow Outcomes Scale.*

[b]*Aneurysms by location: anterior cerebral artery (A1, anterior communicating artery, A2 and pericallosal aneurysms); middle cerebral artery; internal carotid artery (anterior choroidal artery, posterior communicating artery, and carotid terminus aneurysms); posterior circulation (vertebral artery, basilar artery, posterior inferior cerebellar artery, anterior inferior cerebellar artery and superior cerebellar artery aneurysms).*

We evaluated the predictors of discharge to home after admission for aneurysmal subarachnoid hemorrhage, to determine the impact of nimodipine compliance on outcomes at the time of discharge. Our study demonstrates that, along with age and WFNS grade, compliance with scheduled nimodipine is an independent predictor of clinical outcome. We found that patients who missed one or more doses of nimodipine experienced a significantly worse clinical outcome. The recent study by Sandow et al. (45) compared outcomes in patients who received all doses of nimodipine to those who received 30 mg every 4 h or

**TABLE 3** | Adjusted odds ratios of Discharge to Home Among Patients with Subarachnoid Hemorrhage from Brain Aneurysm Rupture 2008–2015, $n = 118$.

| | Odds ratio (95% confidence interval) |
|---|---|
| **Unadjusted association** | |
| Nimodipine compliance | |
| At least one dose held | Reference |
| At least one dose split | 3.41 (0.59, 19.62) |
| All doses received | 5.12 (1.71, 15.33) |
| **Adjusted association** | |
| Nimodipine compliance | |
| At least one dose held | Reference |
| At least one dose split | 3.99 (0.54, 29.60) |
| All doses received | 5.20 (1.46, 18.56) |
| Age, per year | 0.95 (0.91, 0.98) |
| WFNS | |
| 1 | Reference |
| 2 | 0.89 (0.28, 2.79) |
| 3, 4 | 0.16 (0.05, 0.50) |
| 5 | 0.12 (0.02, 0.70) |

*WFNS, World Federation of Neurosurgical Societies Scale.*

**TABLE 4** | Sensitivity analyses.[a]

| | Odds ratio (95% confidence interval) |
|---|---|
| **Nimodipine compliance** | |
| At least one dose held | Reference |
| All doses received or at least one split | 4.86 (1.58, 14.93) |
| **Nimodipine compliance** | |
| At least one dose split or held | Reference |
| All doses received | 4.71 (1.33, 16.63) |

[a]*Adjusted for recoded World Federation of Neurosurgical Societies Scale and age.*

underwent early cessation of nimodipine due to its hypotensive effects. They found that only 43.6% of patients completed a full 14-day course of nimodipine without dose reduction or early cessation. Patients with dose reductions or early cessation experienced greater rates of angiographic vasospasm and cerebral ischemia and were found to have unfavorable clinical outcomes on multivariate analysis.

Various systemic effects, primarily catecholamine-mediated, have been described in association with subarachnoid hemorrhage (46). However, a link between these physiologic derangements and an increased sensitivity to nimodipine remains unproven. While patients with higher grades of subarachnoid hemorrhage may have a greater impairment of cerebral autoregulatory capacity in the days following the ictus, a clear explanation for a greater sensitivity to nimodipine's hypotensive effect is lacking (47–51). Many patients with high Hunt and Hess subarachnoid hemorrhage exhibit increased cardiac troponin I release, regional wall motion abnormalities on echocardiogram, and are more likely to require vasopressor/inotropic infusions (52). Reversible neurogenic myocardial "stunning" related to aneurysmal subarachnoid hemorrhage leads to an increased propensity to delayed ischemic neurologic deterioration related to vasospasm (53), theoretically making compliance with the administration of nimodipine more important.

Although many of our findings are consistent with those of Sandow et al. (45), there were some differences. Some of our patients received 30 mg every 2 h, whereas patients in the Sandow et al. study received doses of 30 mg every 4 h. Sandow et al. determined that the vasopressor dose during the first 14 days was an independent predictor of outcome on multivariable analysis, while we did not include this parameter in our final logistic regression model. Perhaps most notable, our patient's experienced lower rates of cerebral infarction across all dosing groups, which may be attributed to unknown patient characteristics or, as previously reported (36), to the use of a continuous low-dose heparin infusion in our patients.

This study has several limitations, including its retrospective nature and modest sample size. While we stratified patients based on the clinical and radiographic severity of hemorrhage, our classification of patients may not fully account for unmeasured markers of severity, such as concomitant cardiac dysfunction or infection/sepsis, which may confound outcomes data. The study of patients who were all treated with a low-dose IV heparin infusion may limit the generalizability of our findings. Causality remains enigmatic—it is not known whether forcing full compliance would yield more favorable outcomes, or whether patients intolerant to nimodipine, regardless of treatment, are destined to poor outcomes. Regardless, our results are consistent with recent reports that highlight the negative effects of missing nimodipine doses (45). Prospective, randomized-controlled trials will be required to advance understanding of the effects of nimodipine compliance and of combined therapeutic agents in subarachnoid hemorrhage.

## CONCLUSION

Nimodipine is the only agent that has consistently been shown to improve outcomes in patients with aneurysmal subarachnoid hemorrhage. From a practical standpoint, however, the hypotensive effect of nimodipine limits compliance with scheduled dosing. Our study reinforces the importance of nimodipine compliance and its effect on outcomes in aneurysmal subarachnoid hemorrhage, as it was one of three statistically significant predictors of discharge to home.

## ETHICS STATEMENT

This study was conducted under the approval of the Institutional Review Board at the University of Maryland School of Medicine. All procedures performed involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

## AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the following: study design; data acquisition, analysis, and interpretation; manuscript preparation and final manuscript approval.

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# Amino Acids in Cerebrospinal Fluid of Patients with Aneurysmal Subarachnoid Haemorrhage: An Observational Study

*Bartosz Sokół[1]*, Bartosz Urbaniak[2], Norbert Wąsik[1], Szymon Plewa[2], Agnieszka Klupczyńska[2], Roman Jankowski[1], Barbara Więckowska[3], Robert Juszkat[4] and Zenon Kokot[2]*

[1] Department of Neurosurgery, Poznan University of Medical Sciences, Poznan, Poland, [2] Faculty of Pharmacy, Department of Inorganic and Analytical Chemistry, Poznan University of Medical Sciences, Poznan, Poland, [3] Department of Computer Science and Statistics, Poznan University of Medical Sciences, Poznan, Poland, [4] Department of General and Interventional Radiology, Poznan University of Medical Sciences, Poznan, Poland

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*J. Marc Simard, University of Maryland, Baltimore, United States*

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***Correspondence:**
*Bartosz Sokół*
*bartosz.sokol@ump.edu.pl*

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**Background:** The authors are aware of only one article investigating amino acid concentrations in cerebrospinal fluid (CSF) in patients with ruptured intracranial aneurysms, and this was published 31 years ago. Since then, both management of subarachnoid haemorrhage (SAH) and amino acid assay techniques have seen radical alterations, yet the pathophysiology of SAH remains unclear.

**Objective:** To analyse the pattern of concentrations of amino acids and related compounds in patients with different outcomes following aneurysmal SAH.

**Methods:** 49 CSF samples were collected from 23 patients on days 0–3, 5, and 10 post-SAH. Concentrations of 33 amino acids and related compounds were assayed by liquid chromatography tandem mass spectrometry in patients with good [Glasgow Outcome Scale (GOS) 1–3] and poor (GOS 4–5) outcome.

**Results:** Of the 33 compounds assayed, only hydroxyproline and 3-aminoisobutyric acid appeared not to increase significantly following SAH. In poor outcome patients, we found significantly higher concentrations of aspartic acid ($p = 0.038$), glutamic acid ($p = 0.038$), and seven other compounds on days 0–3 post-SAH; glutamic acid ($p = 0.041$) on day 5 post-SAH, and 2-aminoadipic acid ($p = 0.033$) on day 10 post-SAH. The most significant correlation with GOS at 3 months was found for aminoadipic acid on day 10 post-SAH (cc = −0.81).

**Conclusion:** Aneurysmal rupture leads to a generalised increase of amino acids and related compounds in CSF. The patterns differ between good and poor outcome cases. Increased excitatory amino acids are strongly indicative of poor outcome.

**Keywords: subarachnoid haemorrhage, amino acids, early brain injury, delayed cerebral ischaemia, biomarkers**

## INTRODUCTION

Subarachnoid hemorrhage (SAH) due to ruptured intracranial aneurysms is a life-threatening condition with an annual incidence of 2–22.5/100,000 population per annum. The average annual attack rate per 100,000 population for men and women aged 25–64 years in Poland is 10.9 and

9.1, respectively, and the 28 day fatality rates 39 and 44% (1, 2). Cumulative morbidity and mortality following SAH remain high, despite the considerable efforts of neuroclinicians worldwide (3, 4). Traditionally, rebleeding and cerebral vasospasm have been regarded as the main causes of poor outcome in these cases (5). Although cerebral vasospasm has been extensively studied, and subjected to numerous drug trials during the past several decades, the outcome appears not to have been improved by its reversal (2, 6, 7). Several recent studies indicate that early brain injury (EBI), which occurs during the 24–72 h following aneurysmal rupture, makes a significant contribution to patient outcome, and may be responsible for the detrimental effects seen in patients after SAH (3, 8–10). EBI was first reported by Kusaka et al. (11). Since then, the knowledge of mechanisms involved in EBI significantly progressed, but it still warrants further investigation (12–20). During EBI, the central nervous system (CNS) suffers from "primary" insults (involving acute changes of intracranial pressure, cerebral perfusion pressure, and cerebral blood flow with vascular constriction and obstruction of the microcirculation), and "secondary" ischaemic processes (including anaerobic cellular respiration, energy depletion, impaired protein synthesis, excitotoxicity, free radical attack, neuronal stress, and DNA damage, leading to apoptosis and necrosis) (16). Many of these processes may potentially be initiated, mediated, or terminated by amino acids and related compounds. Von Holst and Hagenfeldt in 1985 appear to be the only group to have demonstrated increased levels of amino acids in cerebrospinal fluid (CSF) after SAH, and proposed mechanisms leading to it (21). Since then, no new studies analysing CSF amino acids involved in SAH has been published. Our present aim is to look into the role of amino acids and related compounds potentially involved in the process of brain injury following SAH. Determination of amino acid levels in CSF samples may provide a means of determining prognosis at an early stage and extending the knowledge about the pathophysiology of SAH.

# MATERIALS AND METHODS

## Ethics and Consent

This is a prospective observational study conducted in a single medical centre in accordance with the Declaration of Helsinki. The local bioethics committee approved the study protocol, consenting protocol, and consent forms. Patients were assessed by two specialists (neurosurgeon and anaesthesiologist) as to their ability to give informed consent. Depending on this assessment, either the patient or next of kin gave consent for entry to the study and use a blinded medical data for analysis and further publication.

## Population, Inclusion and Exclusion Criteria

132 patients with SAH [confirmed by non-contrast computed tomography (CT)] were referred to our department during study recruitment period (from May 2015 to October 2016). Inclusion criteria were as follows: (1) aneurysmal SAH treated endovascularly <24 h post rupture, (2) external ventricular drainage (EVD) placed <48 h post rupture. The aim was early prevention of rebleeding while managing acute hydrocephalus (HCP), but avoiding the trauma associated with open surgery. Exclusion criteria for the study were as follows: (1) history of CNS disease (meningitis, stroke), (2) active CNS infection, (3) active systemic disease (diabetes mellitus, rheumatoid arthritis, malignancy, cirrhosis, renal failure), (4) age below 18, and (5) pregnancy. Conditions with potential impact on CSF homeostasis as well as subpopulations with distinct SAH features were not enrolled. Control CSF was obtained during spinal anaesthesia from age- and sex-matched patients with a negative history of CNS diseases.

## Management, Definitions, and End Points

On admission, the clinical status was assessed using GCS and specific SAH grading scales [Hunt and Hess (HH), World Federation of Neurosurgical Societies (WFNS)]. An initial head CT scan was used to confirm SAH and assess the presence of HCP. EVD was placed secondary to endovascular treatment in patients with GCS score below 15 and: (1) relative bicaudate index >1, (2) focal dilation of ventricular system due to obstruction, or (3) thick intraventricular blood clot. A second CT scan was performed within 24 h of aneurysmal occlusion and EVD placement to assess any procedure related brain injury. Patients received a continuous infusion of nimodipine for at least 10 days, hypotension was avoided using vasopressors, and euvolemia was maintained. Induced hypertension (20–30% above baseline levels) was used to treat patients diagnosed with delayed cerebral ischaemia (DCI), based on the appearance of a new focal deficit, or a drop of at least two points on the GCS lasting at least 2 h after the exclusion of systematic causes EVD infection screening involved CSF cell count at least twice per patient, and CSF culture at least once on day 10 post-SAH. The primary end point was the treatment outcome assessed at 3 months using the Glasgow Outcome Scale (GOS) (22). Patients were divided into two groups according to GOS. Good outcome consisted of those with no disability, moderate disability, and severe disability (GOS grades 5, 4, and 3); poor outcome were those with persistent vegetative state or death (GOS grades 2 and 1). DCI related infarction was defined as a new cerebral infarction identified on a head CT scan within 6 weeks of rupture and not present on the immediate posttreatment scan [as proposed by Vergouwen et al. (23)].

## Plasma Assays and CSF Sample Collection

Haemoglobin, white blood cell (WBC) count, C-reactive protein (CRP) level, and fibrinogen level were assessed daily. Automatic analysers XT 2000i (Sysmex, Japan), Cobas 6000 (Roche Diagnostic, USA), and ACL TOP 500 (Instrumentation Laboratory, Italy) were used for measurements. CSF samples were collected from the EVD at three time points, on post-SAH days 0–3, 5, and 10. Each CSF sample was centrifugated and stored at −80°C until assayed.

## Determination of Free Amino Acid Profiles

The applied methodology was based on an aTRAQ™ kit (Sciex, Framingham, MA, USA). The detailed description of a sample preparation procedure as well as liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS) parameters

have been described in our previous report (24). The sample preparation comprised the following steps: protein precipitation by 10% sulphosalicyclic acid, dilution with borate buffer, amino acids labelling with aTRAQ™ reagent, and addition of internal standard mixture. Labelling efficiency was confirmed using norleucine (contained in sulphosalicylic acid solution) and norvaline (contained in borate buffer). LC–MS/MS assays were carried out on a high-performance liquid chromatography instrument 1260 Infinity (Agilent Technologies, Santa Clara, CA, USA) coupled with 4000 QTRAP triple quadrupole mass spectrometer (Sciex, Framingham, MA, USA). Chromatographic separation of amino acids was performed using a Sciex C18 column (4.6 mm × 150 mm, 5 μm) and a flow rate of 800 μL/min in a gradient elution mode. Solvent A was water and solvent B was methanol, both with 0.1% formic acid and 0.01% heptafluorobutyric acid. The injection volume was 2 μL. The mass spectrometer was equipped with an electrospray ionisation source and operated in positive ionisation mode. For detection and quantification of amino acids, a highly selective schedule multiple reaction monitoring mode was used. Data acquisition and processing were performed with Analyst 1.5 software (Sciex, Framingham, MA, USA). The applied methodology is suitable for simultaneous determination of a wide range of 33 free amino acids, both proteinogenic and non-proteionogenic, with high sensitivity and specificity in time below 20 min (24–26). Due to simplification used in this article, *o*-phosphoethanolamine and ethylamine are counted to amino acids, yet in fact they are amino acids derivatives.

## Statistical Analysis

Statistical analysis was performed using STATISTICA 10 (Stat Soft Inc., Tulsa, OK, USA). Values for normally distributed numerical data have been expressed as mean and SDs; for ordinal or non-normally distributed numerical data as median and interquartile range, and for categorical data as counts and percentages. The normality of data distribution was assessed using the Shapiro–Wilk test. Amino acids levels are presented on figures as median and interquartile range since in nearly all cases they do not show normal distribution. Comparisons were made by using (1) Mann–Whitney test, (2) Student's *t*-test, (3) Friedman test with Conover–Iman *post hoc*, and (4) repeated measures ANOVA test with Fisher *post hoc*. The correlations were assessed by Spearman's test, and correlation coefficient (cc) >0.6 (cc < −0.6) was considered significant. A value of $p < 0.05$ was considered statistically significant when comparing.

# RESULTS

The concentrations of the 33 compounds were established for 49 samples derived from 23 SAH patients (**Table 1**), and 25 samples collected from 25 control patients. Assays were available from 22 samples at days 0–3 post-SAH, 15 from days 5, and 12 from day 10. Rupture of an aneurysm led to a significant elevation of 27 of the 33 compounds in the CSF at days 0–3 post-SAH (**Table 2**). On days 0–3 post-SAH, the six exceptions were ethylamine ($p = 0.798$), gamma-aminobutyric acid ($p = 0.699$), 3-amino-isobutyric acid ($p = 0.668$), hydroxyproline ($p = 0.164$), threonine ($p = 0.079$), and arginine ($p = 0.062$). On day 5 post-SAH, three compounds showed no significant difference—aspartic acid ($p = 0.175$), 3-amino-isobutyric acid ($p = 0.563$), and hydroxyproline ($p = 0.658$). On day 10 post-SAH, there were four—aspartic acid ($p = 0.054$), 3-amino-isobutyric acid ($p = 0.806$), hydroxyproline ($p = 0.289$), and ethylamine ($p = 0.292$). Thus, hydroxyproline and 3-amino-isobutyric acid are the only 2 of the 33 substances assayed which appear to show no increase following SAH.

Patients were now divided into two groups as good and poor outcomes as defined above (**Table 3**). On days 0–3 post-SAH, concentrations of nine amino acids were significantly higher in patients with poor outcome than those with good outcome: taurine ($p = 0.038$), aspartic acid ($p = 0.038$), citrulline ($p = 0.035$), glutamic acid ($p = 0.038$), gamma-amino-butyric acid ($p = 0.043$), 3-methyl-histidine ($p = 0.01$), ornithine ($p = 0.033$), cystathionine ($p = 0.01$), and isoleucine ($p = 0.045$). WBC level ($p = 0.01$) also differentiated these two groups (**Figure 1**). On day 5 post-SAH, glutamic acid ($p = 0.041$) was the only amino acid showing significantly higher levels in the poor outcome group.

**TABLE 1** | Baseline characteristics of study patients.

| | |
|---|---|
| Male | 12 (52%) |
| Age (years) | 57.26 ± 14.78 |
| **Aneurysm location** | |
| Anterior communicating artery | 8 (35%) |
| Middle cerebral artery | 6 (26%) |
| Anterior cerebral artery | 3 (13%) |
| Basilar artery | 3 (13%) |
| Internal carotid artery | 1 (4%) |
| Posterior cerebral artery | 1 (4%) |
| Posterior inferior cerebellar artery | 1 (4%) |
| Aneurysmal size (mm) | 4.74 ± 1.82 |
| Cerebral infarction due to DCI on CT | 15 (65%) |
| Intracerebral haemorrhage on CT | 15 (65%) |
| Intraventricular blood on CT | 21 (91%) |
| Fisher CT score | 4 (4–4) |
| Modified Fisher CT score | 4 (3–4) |
| WFNS score on admission | 4 (3–5) |
| HH score on admission | 4 (3–5) |
| GCS on admission | 5 (4–12) |

| | On admission | Post-SAH day 5 | Post-SAH day 10 |
|---|---|---|---|
| WBC count ($10^6$/mm$^3$) | 13.69 ± 5.4 | 11.86 ± 5.7 | 12.51 ± 4.3 |
| CRP level (mg/L) | 119.50 ± 79.5 | 89.42 ± 54.4 | 78.70 ± 119 |
| Fibrinogen (mg/dL) | 446.70 ± 151 | 608.20 ± 205 | 592.90 ± 249 |
| Hgb (g/dL) | 16.40 ± 17.41 | 11.31 ± 1.9 | 10.93 ± 1.3 |

| **Treatment outcome (according to GOS at 3 months)** | |
|---|---|
| Good recovery (score of 5) | 6 (26%) |
| Moderate disability (score of 4) | 2 (9%) |
| Severe disability (score of 3) | 2 (9%) |
| Persistent vegetative state (score of 2) | 5 (22%) |
| Death (score of 1) | 8 (35%) |

*Data presented as mean ± SD; median (interquartile range) or count (percentage). CRP, C-reactive protein; CT, computed tomography; DCI, delayed cerebral ischaemia; GCS, Glasgow Coma Scale; GOS, Glasgow Outcome Scale; HH, Hunt and Hess scale; Hgb, plasma haemoglobin; WBC, white blood cell; WFNS, World Federation of Neurosurgical Societies scale.*

**TABLE 2** | Differences in cerebrospinal fluid amino acid level at days 0–3, 5, and 10 post-SAH in healthy individuals (control group) and subarachnoid haemorrhage (SAH) patients (study group).

| Amino acid | Control group | Study group, day 0–3 post-SAH | Control group vs study group, days 0–3 post-SAH. *p* value | Study group, day 5 post-SAH | Control group vs study group, day 5 post-SAH. *p* value | Study group, day 10 post-SAH | Control group vs study group, day 10 post-SAH. *p* value |
|---|---|---|---|---|---|---|---|
| O-phosphoethanolamine (μM) | 3.00 | 5.95 | **<0.01** | 5.15 | **<0.001** | 6.60 | **<0.001** |
| Ethylamine (μM) | 9.80 | 9.40 | 0.798 | 9.65 | **<0.001** | 11.10 | 0.292 |
| Taurine (μM) | 7.00 | 16.00 | **<0.001** | 14.30 | **<0.001** | 10.75 | **<0.01** |
| Asparagine (μM) | 5.70 | 10.35 | **0.01** | 19.50 | **<0.001** | 21.75 | **<0.001** |
| Serine (μM) | 24.20 | 55.70 | **<0.001** | 75.40 | **<0.001** | 83.75 | **<0.001** |
| Glycine (μM) | 8.90 | 38.75 | **<0.001** | 34.85 | **<0.001** | 35.20 | **<0.001** |
| Hydroxyproline (μM) | 0.60 | 1.00 | 0.164 | 1.35 | 0.658 | 1.35 | 0.289 |
| Glutamine (μM) | 407.60 | 520.65 | **<0.01** | 791.10 | **<0.001** | 796.30 | **<0.001** |
| Aspartic acid (μM) | 0.50 | 1.55 | **<0.001** | 0.80 | 0.175 | 0.95 | 0.054 |
| Citruline (μM) | 1.60 | 2.75 | **0.011** | 2.55 | **<0.01** | 3.30 | **<0.01** |
| Threonine (μM) | 24.80 | 29.05 | 0.079 | 56.10 | **<0.001** | 63.40 | **<0.001** |
| Beta-alanine (μM) | 20.30 | 22.00 | **0.040** | 29.00 | **<0.01** | 27.30 | **<0.01** |
| Alanine (μM) | 35.00 | 88.05 | **<0.001** | 114.55 | **<0.001** | 122.55 | **<0.001** |
| Glutamic acid (μM) | 1.20 | 5.40 | **<0.001** | 3.00 | **< 0.001** | 2.35 | **<0.01** |
| Histidine (μM) | 11.40 | 28.20 | **<0.001** | 43.35 | **<0.001** | 40.20 | **<0.001** |
| 3-Methylhistidine (μM) | 0.50 | 1.00 | **<0.01** | 1.40 | **<0.001** | 1.05 | **<0.01** |
| 2-Aminoadipic acid (μM) | 0.00 | 1.65 | **<0.001** | 2.10 | **<0.001** | 1.45 | **<0.001** |
| Gamma-aminobutyric acid (μM) | 0.40 | 0.50 | 0.699 | 0.30 | **0.012** | 0.20 | 0.051 |
| 3-Aminoisobutyric acid (μM) | 0.50 | 0.30 | 0.668 | 0.30 | 0.563 | 0.35 | 0.806 |
| 2-Aminobutyric acid (μM) | 3.20 | 6.20 | **<0.01** | 6.60 | **<0.001** | 7.30 | **<0.001** |
| Arginine (μM) | 15.70 | 19.25 | 0.062 | 26.60 | **<0.001** | 24.35 | **<0.01** |
| Proline (μM) | 0.80 | 11.80 | **<0.001** | 13.95 | **<0.001** | 16.15 | **<0.001** |
| Ornithine (μM) | 4.30 | 19.80 | **<0.001** | 17.95 | **<0.001** | 17.75 | **<0.001** |
| Cystathionine (μM) | 0.30 | 2.05 | **<0.001** | 1.05 | **<0.001** | 1.40 | **<0.001** |
| Cysteine (μM) | 0.40 | 2.05 | **<0.001** | 1.75 | **<0.001** | 1.65 | **<0.001** |
| Lysine (μM) | 22.50 | 53.80 | **<0.001** | 73.05 | **<0.001** | 72.40 | **<0.001** |
| Methionine (μM) | 3.10 | 6.20 | **<0.01** | 11.70 | **<0.001** | 13.65 | **<0.001** |
| Valine (μM) | 16.20 | 38.65 | **<0.01** | 66.20 | **<0.001** | 76.30 | **<0.001** |
| Tyrosine (μM) | 7.80 | 26.70 | **<0.001** | 36.20 | **<0.001** | 37.30 | **<0.001** |
| Isoleucine (μM) | 4.40 | 9.65 | **0.001** | 13.05 | **<0.001** | 15.05 | **<0.001** |
| Leucine (μM) | 11.40 | 29.20 | **<0.001** | 43.50 | **<0.001** | 56.80 | **<0.001** |
| Phenylalanine (μM) | 9.40 | 26.45 | **<0.001** | 43.25 | **<0.001** | 44.45 | **<0.001** |
| Tryptophan (μM) | 1.90 | 9.70 | **<0.001** | 13.60 | **<0.001** | 12.85 | **<0.001** |

*Median levels of the amino acids are presented in the table. p values were calculated either by Mann–Whitney or Student's t-test. Amino acids increasing significantly during SAH (on days 0–3, 5, or 10) are shown in bold.*

At this stage, CRP ($p = 0.020$) and WBC ($p = 0.044$) were also significantly higher in the poor outcome group (**Figure 2**). On day 10 post-SAH, 2-amino-adipic acid ($p = 0.033$) and fibrinogen ($p = 0.014$) were the only parameters showing significantly higher levels in the poor outcome group (**Figure 3**).

In the course of the study, it was possible to undertake assays at all three time points in 10 of the subjects; 7 of these were in the good outcome group. In this group, the Friedman test with Conover–Iman *post hoc*, or ANOVA test with Fisher *post hoc*, revealed significant changes in the concentrations of 18 of the compounds tested (**Table 4**). The number of poor outcome patients was too small to carry out such testing.

The levels of four of the compounds tested showed a significant correlation with GOS at 3 months. These were 2-amino-adipic acid on day 10 post-SAH (cc = −0.81), cystathionine on day 5 post-SAH (cc = −0.72) and day 10 post-SAH (cc = −0.67), 3-methylhistidine on days 0–3 post-SAH (cc = −0.64), and *o*-phosphoethanolamine on day 10 post-SAH (cc = −0.62). As might be expected, there was a correlation between all three admission assessments (WFNS, HH, and GCS) and outcome. There were also correlations between outcome and CRP on days 5 and 10 post-SAH (cc = −0.64 and cc = −0.79, respectively) as well as fibrinogen level on day 10 post-SAH (cc = −0.97) (**Figure 4**).

## DISCUSSION

In this study, we have analysed the profiles of amino acids and related compounds in the CSF of patients following SAH. The most significant findings are as follows:

(1) Increase in 31 of 33 compounds in the days following SAH, with 26 increasing in the first 3 days after rupture
(2) Significant increase in 18 of these compounds between days 0–3 and days 5 and 10
(3) Higher levels of excitatory amino acids (EAAs) (glutamic acid, aspartic acid, and 2-amino-adipic acid) appear to predict a poor outcome.

As far as we are aware, it is 31 years since an article investigating amino acids in SAH has been published. In view of the paucity

**TABLE 3** | Differences in monitored parameters at days 0–3, 5, and 10 post-SAH in patients with good (GO-SAH) and poor (PO-SAH) treatment outcome.

| Monitored parameter | Days 0–3 post-SAH | | | Day 5 post-SAH | | | Day 10 post-SAH | | |
|---|---|---|---|---|---|---|---|---|---|
| | GO-SAH | PO-SAH | GO-SAH vs PO-SAH. *p* value | GO-SAH | PO-SAH | GO-SAH vs PO-SAH. *p* value | GO-SAH | PO-SAH | GO-SAH vs PO-SAH. *p* value |
| C-reactive protein (mg/L) | 125.00 | 130.00 | 0.715 | 50.70 | 115.80 | **0.020** | 22.40 | 109.70 | 0.139 |
| White blood cell count ($10^6$/$mm^3$) | 9.86 | 17.47 | **<0.001** | 10.57 | 15.03 | **0.044** | 9.66 | 16.84 | 0.445 |
| Haemoglobin (g/dL) | 12.10 | 13.10 | 0.212 | 11.30 | 10.90 | 0.262 | 10.60 | 11.50 | 0.672 |
| Temperature (°C) | 37.00 | 37.00 | 0.736 | 37.40 | 37.20 | 0.273 | 36.90 | 35.70 | 0.512 |
| Fibrinogen (mg/dL) | 485.00 | 550.00 | 0.879 | 509.00 | 676.00 | 0.257 | 428.00 | 698.00 | **0.014** |
| O-phosphoethanolamine (μM) | 4.10 | 6.80 | 0.161 | 4.90 | 7.70 | 0.161 | 5.95 | 8.50 | 0.019 |
| Taurine (μM) | 13.10 | 19.40 | **0.038** | 14.60 | 13.40 | 0.514 | 12.20 | 7.35 | 0.138 |
| Asparagine (μM) | 9.00 | 11.50 | 0.256 | 30.20 | 12.50 | 0.397 | 22.90 | 20.45 | 0.423 |
| Serine (μM) | 45.10 | 76.50 | 0.161 | 77.00 | 61.40 | 0.947 | 79.50 | 94.75 | 0.503 |
| Glycine (μM) | 29.60 | 41.00 | 0.182 | 30.00 | 43.00 | 0.204 | 35.20 | 35.65 | 0.671 |
| Hydroxyproline (μM) | 0.70 | 1.30 | 0.066 | 1.70 | 0.80 | 0.518 | 1.35 | 1.10 | 0.793 |
| Ethylaminev (μM) | 5.80 | 15.80 | 0.102 | 7.90 | 12.10 | 0.134 | 8.85 | 14.50 | 0.35 |
| Glutamine (μM) | 489.90 | 602.50 | 0.182 | 837.30 | 505.10 | 0.585 | 852.50 | 715.60 | 0.483 |
| Aspartic acid (μM) | 0.80 | 2.20 | **0.038** | 0.40 | 1.00 | 0.071 | 0.95 | 1.30 | 0.551 |
| Citruline (μM) | 1.90 | 5.90 | **0.035** | 2.50 | 3.10 | 0.396 | 3.30 | 3.30 | 1 |
| Threonine (μM) | 25.90 | 50.00 | 0.35 | 86.30 | 34.20 | 0.327 | 63.40 | 57.95 | 0.298 |
| Beta-alanine (μM) | 28.30 | 22.00 | 0.07 | 30.00 | 28.60 | 0.497 | 26.85 | 27.85 | 0.893 |
| Alanine (μM) | 70.70 | 138.10 | 0.109 | 115.50 | 113.60 | 0.447 | 120.30 | 141.70 | 0.954 |
| Glutamic acid (μM) | 3.40 | 9.30 | **0.038** | 2.70 | 5.80 | **0.024** | 2.00 | 17.10 | 0.269 |
| Histidine (μM) | 18.90 | 38.10 | 0.256 | 63.60 | 23.70 | 0.711 | 43.55 | 37.45 | 0.753 |
| 3-Methylhistidine (μM) | 0.70 | 1.50 | **<0.01** | 1.80 | 1.20 | 0.958 | 1.35 | 1.05 | 0.733 |
| 2-Aminoadipic acid (μM) | 1.30 | 1.70 | 0.076 | 1.50 | 4.10 | 0.071 | 1.30 | 2.45 | **0.033** |
| Gamma-aminobutyric acid (μM) | 0.30 | 0.90 | **0.043** | 0.30 | 0.30 | 0.932 | 0.35 | 0.15 | 0.266 |
| 3-Aminoisobutyric acid (μM) | 0.20 | 0.40 | 0.066 | 0.30 | 0.30 | 0.958 | 0.35 | 0.45 | 0.93 |
| 2-Aminobutyric acid | 4.60 | 8.20 | 0.088 | 11.40 | 5.50 | 0.542 | 10.75 | 6.50 | 0.124 |
| Arginine (μM) | 18.50 | 22.30 | 0.182 | 30.90 | 26.00 | 0.525 | 24.35 | 20.45 | 0.21 |
| Proline (μM) | 4.50 | 14.80 | 0.204 | 18.70 | 10.90 | 0.672 | 16.15 | 18.40 | 0.759 |
| Ornithine (μM) | 7.60 | 27.50 | **0.033** | 17.30 | 28.30 | 0.09 | 17.35 | 33.05 | 0.552 |
| Cystathionine (μM) | 0.80 | 2.80 | **<0.01** | 1.00 | 1.80 | 0.089 | 1.30 | 1.50 | 0.199 |
| Cysteine (μM) | 1.60 | 2.30 | 0.141 | 1.50 | 2.20 | 0.243 | 1.95 | 1.40 | 0.329 |
| Lysine (μM) | 43.00 | 74.50 | 0.095 | 87.10 | 55.90 | 0.341 | 72.40 | 72.60 | 0.612 |
| Methionine (μM) | 4.60 | 12.30 | 0.204 | 16.20 | 7.70 | 0.491 | 13.65 | 13.70 | 0.687 |
| Valine (μM) | 31.80 | 62.40 | 0.109 | 102.50 | 33.60 | 0.397 | 87.75 | 72.20 | 0.377 |
| Tyrosine (μM) | 23.00 | 32.50 | 0.083 | 36.60 | 35.80 | 0.876 | 38.60 | 37.30 | 0.676 |
| Isoleucine (μM) | 6.00 | 13.00 | **0.045** | 17.60 | 11.80 | 0.597 | 15.05 | 13.70 | 0.45 |
| Leucine (μM) | 22.90 | 39.70 | 0.109 | 80.30 | 29.80 | 0.397 | 60.95 | 50.55 | 0.532 |
| Phenylalanine (μM) | 21.20 | 38.80 | 0.062 | 51.30 | 32.40 | 0.665 | 45.40 | 44.45 | 0.913 |
| Tryptophan (μM) | 8.00 | 12.20 | 0.066 | 13.60 | 13.60 | 0.606 | 12.60 | 13.55 | 0.804 |

*Median levels of the amino acids are presented in the table. p values were calculated either by Mann–Whitney or Student's t-test. Significant differences are shown in bold.*

of publications, knowledge of this subject remains rudimentary. With the introduction of microdialysis, interest in this subject has been renewed since it allows *in vivo* sampling of brain interstitial fluid (27). This method allows continuous monitoring of brain metabolism, but this is limited to the tissue around the probe (28). On the other hand, the commonly available CSF analysis gives a more general picture of conditions in the brain. Physiologically, the exchange between brain interstitial fluid and CSF is bidirectional (29). In mice, levels of amino acids in brain interstitial fluid were found to be approximately 5–10 times lower than in the CSF (30). Although the latest studies of amino acids in SAH have focussed on microdialysis, widespread use of this monitoring technique is limited by its high cost. By contrast, EVD is a relatively inexpensive procedure commonly performed in patients following SAH. In the clinical setting, CSF seems to be a more convenient source for examining biomarkers.

In our patients, rupture of an aneurysm led to an increase in 31 of 33 amino acids and related compounds we assayed. In the study by von Holst et al., there was no increase in taurine levels following SAH. Because the concentration of taurine in whole blood is four to eight times greater than in blood plasma alone, von Holst et al. concluded that red blood cell (RBC) lysis did not contribute to the amino acid concentrations (31). We do not agree with this statement as in our series, the CSF taurine levels increased by a factor of 2. RBC lysis begins within 2–4 h of SAH and continues at least until the clot has cleared (32, 33). In our opinion, this process contributes to the amino acid levels at every stage following SAH. Microdialysis studies indicate that an early increase of taurine in the brain interstitial fluid is a reliable marker of poor outcome (34, 35). In the both articles, brain cells activated in the course of SAH were indicated as a potential source of taurine in the interstitial fluid. In our series, significantly higher taurine levels were observed on days 0–3 post-SAH in patients with a poor outcome. This observation suggests that taurine may have some value as a clinical marker and encourage further studies. In experimental settings, both detrimental and beneficial roles for taurine have

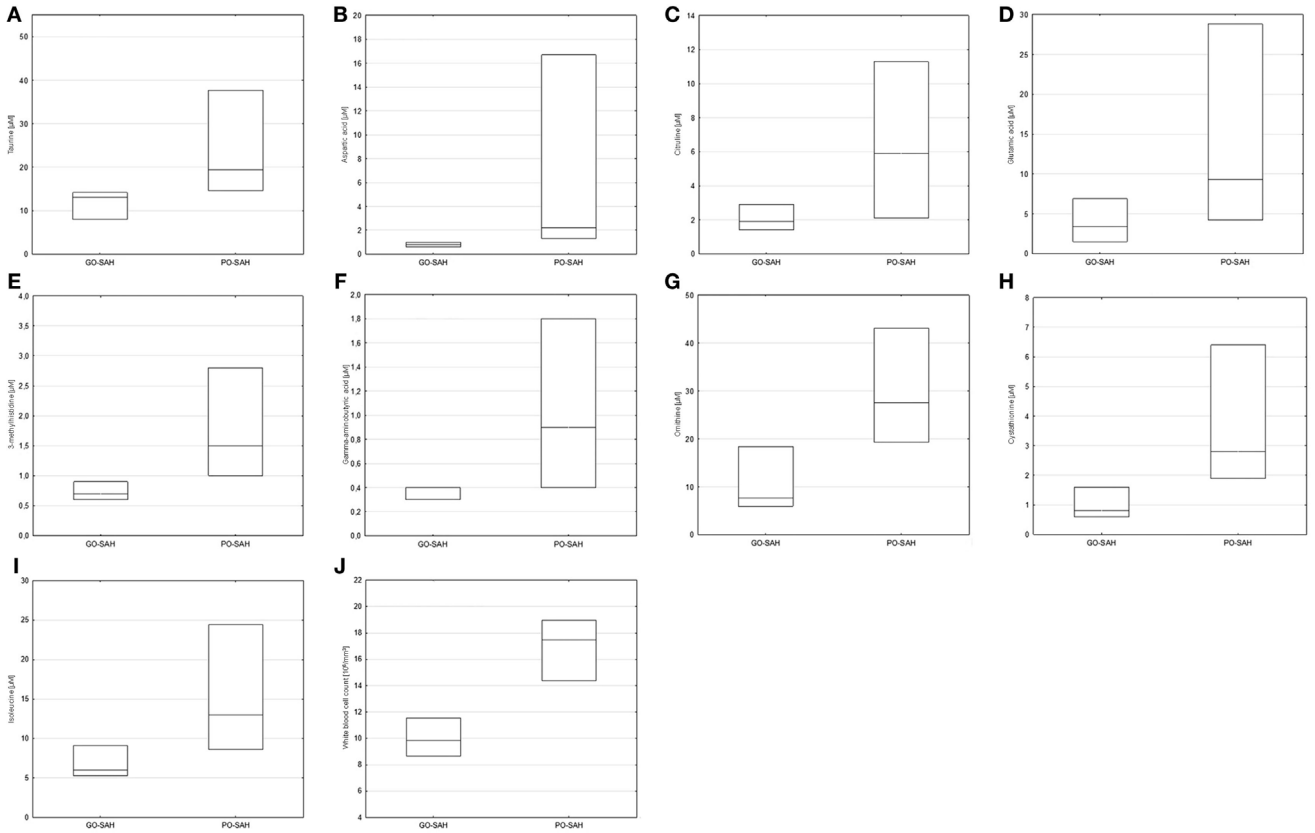


**FIGURE 1** | Significant differences in monitored parameters on days 0–3 post-SAH between patients with good (GO-SAH) and poor (PO-SAH) treatment outcome. Mann–Whitney test revealed significantly higher levels of **(A)** taurine ($p = 0.038$), **(B)** aspartic acid ($p = 0.038$), **(C)** citruline ($p = 0.035$), **(D)** glutamic acid ($p = 0.038$), **(E)** 3-methylhistidine ($p < 0.01$), **(F)** gamma-aminobutyric acid ($p = 0.043$), **(G)** ornithine ($p = 0.033$), **(H)** cystathionine ($p < 0.01$), and **(I)** isoleucine ($p = 0.045$) in patients with poor outcome. Student's *t*-test revealed significantly higher level of white blood cell count ($p < 0.01$) in patients with poor outcome **(J)**. In all cases, median levels and the 25th and 75th percentiles are presented.

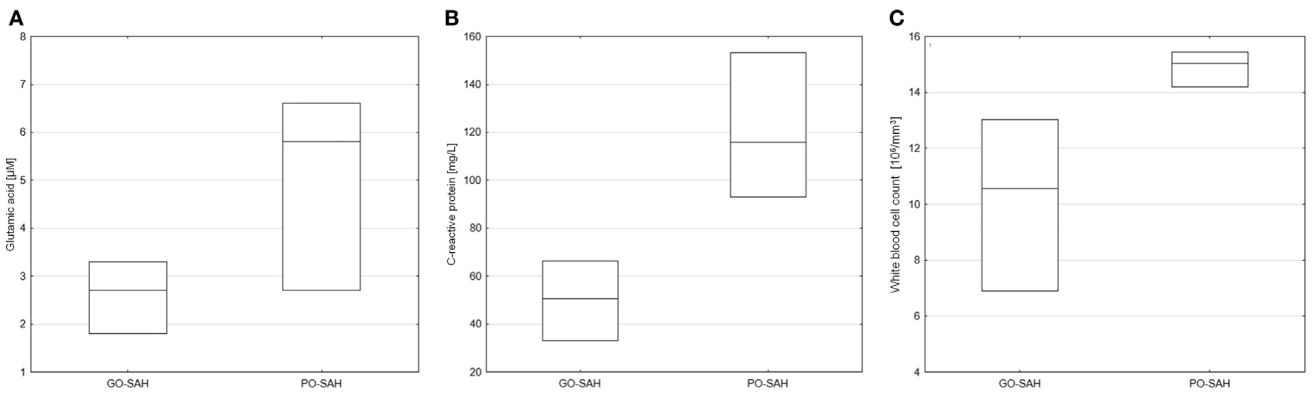


**FIGURE 2** | Significant differences in monitored parameters on day 5 post-SAH between patients with good (GO-SAH) and poor (PO-SAH) treatment outcome. **(A)** Student's *t*-test revealed significantly higher levels of glutamic acid ($p = 0.041$) in patients with poor outcome. Mann–Whitney test revealed significantly higher C-reactive protein level ($p$ 0.020) **(B)** and white blood cell count ($p = 0.044$) **(C)** in patients with poor outcome. In all cases, median levels and the 25th and 75th percentiles are presented.

been described. Kofler et al. have extensively discussed this matter in the context of SAH (34). Our study suggests predominantly harmful effects from taurine.

Among 33 assayed compounds, only 2 did not increase at any stage; these were hydroxyproline and 3-aminoisobutyric acid, neither of which is encoded in the eukaryotic genetic code.

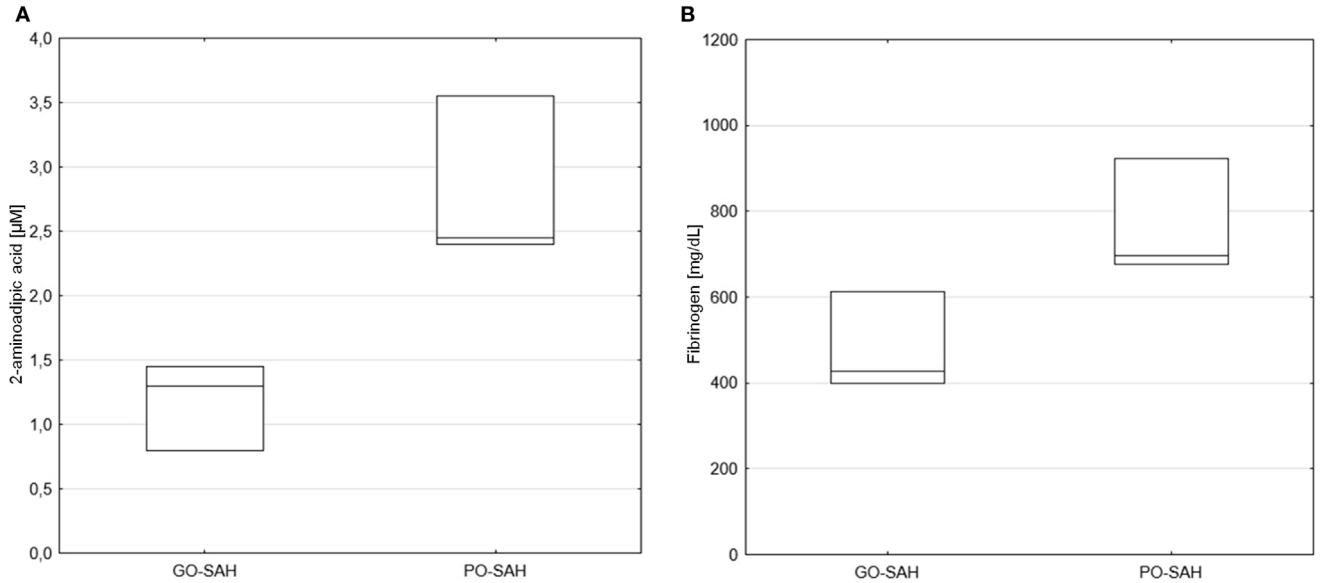


**FIGURE 3** | Significant differences in monitored parameters on day 10 post-SAH between patients with good (GO-SAH) and poor (PO-SAH) treatment outcome. Mann–Whitney test revealed significantly higher 2-aminoadipic acid ($p = 0.033$) **(A)** and fibrinogen ($p = 0.014$) **(B)** levels in patients with poor outcome. Median levels and the 25th and 75th percentiles are presented.

**TABLE 4** | Amino acid level changes in time in good outcome SAH patients with (GO-SAH).

| Monitored parameter | Days 0–3 post-SAH | Day 5 post-SAH | Day 10 post-SAH | Omnibus test *p* value | Days 0–3 vs Day 5 *p* value *post hoc* | Days 0–3 vs Day 10 *p* value *post hoc* | Day 5 vs Day 10 *p* value *post hoc* |
|---|---|---|---|---|---|---|---|
| C-reactive protein (mg/L) | 125.00 | 50.70 | 27.00 | **0.049** | 0.345 | **0.012** | 0.073 |
| White blood cell count ($10^6$/mm$^3$) | 9.86 | 10.57 | 9.34 | 0.631 | | | |
| Haemoglobin (g/dL) | 12.10 | 10.60 | 10.50 | 0.066 | | | |
| Temperature (°C) | 37.00 | 37.30 | 36.90 | 0.382 | | | |
| Fibrinogen (mg/dL) | 485.00 | 442.00 | 423.00 | 0.327 | | | |
| O-phosphoethanolamine (μM) | 6.20 | 5.00 | 5.80 | 0.325 | | | |
| Taurine (μM) | 13.90 | 14.60 | 10.80 | 0.867 | | | |
| Asparagine (μM) | 8.40 | 18.30 | 21.20 | 0.180 | | | |
| Serine (μM) | 45.10 | 73.80 | 76.10 | **0.031** | **0.039** | **0.013** | 0.566 |
| Glycine (μM) | 29.60 | 32.60 | 42.50 | 0.565 | | | |
| Hydroxyproline (μM) | 0.70 | 1.10 | 1.40 | 0.094 | | | |
| Ethylaminev (μM) | 5.70 | 10.10 | 9.80 | **0.039** | 0.078 | **<0.01** | 0.187 |
| Glutamine (μM) | 454.30 | 818.70 | 828.30 | **0.001** | **<0.01** | **<0.001** | 0.565 |
| Aspartic acid (μM) | 0.70 | 0.40 | 1.20 | 0.215 | | | |
| Citruline (μM) | 1.90 | 2.40 | 2.80 | 0.069 | | | |
| Threonine (μM) | 22.90 | 52.50 | 61.20 | **0.012** | 0.128 | **<0.01** | **<0.01** |
| Beta-alanine (μM) | 28.30 | 26.80 | 26.70 | 0.553 | | | |
| Alanine (μM) | 70.70 | 106.20 | 120.30 | 0.124 | | | |
| Glutamic acid (μM) | 4.50 | 2.70 | 2.10 | 0.898 | | | |
| Histidine (μM) | 16.80 | 38.10 | 37.40 | 0.156 | | | |
| 3-Methylhistidine (μM) | 0.60 | 1.50 | 0.90 | 0.369 | | | |
| 2-Aminoadipic acid (μM) | 1.30 | 1.50 | 1.30 | 0.129 | | | |
| Gamma-aminobutyric acid (μM) | 0.30 | 0.20 | 0.50 | 0.633 | | | |
| 3-Aminoisobutyric acid (μM) | 0.20 | 0.30 | 0.30 | 0.215 | | | |
| 2-Aminobutyric acid (μM) | 4.60 | 11.40 | 11.40 | **0.010** | **0.019** | **<0.01** | 0.427 |
| Arginine (μM) | 18.50 | 26.50 | 24.10 | **0.050** | 0.073 | **0.012** | 0.345 |
| Proline (μM) | 3.40 | 11.90 | 15.20 | 0.062 | | | |
| Ornithine (μM) | 7.60 | 17.30 | 14.30 | 0.368 | | | |
| Cystathionine (μM) | 1.00 | 1.00 | 1.20 | 0.648 | | | |
| Cysteine (μM) | 1.60 | 1.70 | 2.10 | 0.368 | | | |

(*Continued*)

**TABLE 4** | Continued

| Monitored parameter | Days 0–3 post-SAH | Day 5 post-SAH | Day 10 post-SAH | Omnibus test *p* value | Days 0–3 vs Day 5 *p* value *post hoc* | Days 0–3 vs Day 10 *p* value *post hoc* | Day 5 vs Day 10 *p* value *post hoc* |
|---|---|---|---|---|---|---|---|
| Lysine (μM) | 43.00 | 83.20 | 70.80 | **0.017** | **0.017** | **<0.01** | 0.735 |
| Methionine (μM) | 4.60 | 10.00 | 11.90 | **0.008** | **0.029** | **<0.01** | 0.200 |
| Valine (μM) | 28.20 | 61.60 | 78.10 | **0.015** | **0.017** | **<0.01** | 0.683 |
| Tyrosine (μM) | 23.00 | 35.10 | 28.50 | **0.020** | **0.023** | **<0.01** | 0.647 |
| Isoleucine (μM) | 5.90 | 11.10 | 13.60 | **0.024** | 0.081 | **<0.01** | 0.219 |
| Leucine (μM) | 22.60 | 39.70 | 60.50 | **0.024** | **0.022** | **0.013** | 0.801 |
| Phenylalanine (μM) | 20.90 | 35.20 | 37.50 | **0.038** | **0.040** | **0.018** | 0.700 |
| Tryptophan (μM) | 8.00 | 11.80 | 11.10 | 0.072 | | | |

*Median levels of the amino acids are presented in the table. Omnibus statistical test (Friedman or repeated measures ANOVA) revealed significant changes in concentration of 13 amino acids (shown in bold). Next, post hoc analysis (Conover–Iman for Friedman test and Fisher for repeated measures ANOVA) was run for those 13 amino acids to specify between what time points (days 0–3, 5, or 10 post-SAH) the differences in concentration were significant (shown in bold).*

Hydroxyproline is produced by hydroxylation of proline and incorporated into collagen protein (36). In patients with blood–CSF barrier dysfunction, hydroxyproline has a smaller biological variation in CSF when compared with other amino acids (37). Hydroxyproline is increased in the blood plasma in Alzheimer's disease, but shows no increase in Parkinson's disease (38, 39). High protein bound of hydroksyproline in human erythrocytes could be an explanation of its elevation absence in CSF following SAH (40). Even less data are available for 3-aminoisobutyric acid, although it is known that it is a product of thymine catabolism and plays a role in fatty acid metabolism (41). The alterations of 3-aminoisobutyric acid could be a potential marker for the monitoring of the blood–brain barrier condition in the future studies (42).

Immediately after aneurysmal rupture, significantly higher levels of nine of the compounds investigated identified the patients with a poorer prognosis. Furthermore, none of the substances showed a significant decrease in CSF following SAH. We suspect that the initial increase is due to extravasated blood, and its extent related to the amount of blood. The main source is likely to be plasma, since the majority of amino acids (except glutamine and glutamic acid) have a CSF:plasma ratio of 0.1–0.2 (43). This is consistent with the fact that patients with a greater volume of subarachnoid blood have a poorer outcome (44).

In 18 of the compounds we investigated, there was a significant increase from days 0–3 post-SAH to days 5 and 10. There are several possible mechanisms for this delayed rise: (1) increased amino acid turnover as a response to injury. Zetterling et al. proposed this mechanism as an explanation for an increase in the concentrations of eight non-transmitter amino acids in brain interstitial fluid (45). (2) Cytokine-stimulated amino acid release. High-mobility group box 1 protein (HMGB1), which is a proinflammatory cytokine [found in CSF of SAH patients (46)], which induces the release of the glutamate analogue from gliosomes (glial resealed subcellular particles) in a concentration-dependent manner (47). Conversely, HMGB1 was found to accumulate in glutamate treated primary cortical culture media, and supernatants collected from these cultures were found to trigger microglial activation (48). (3) Disruption of CSF homeostasis affecting transport with both the bloodstream and the interstitial fluid. (4) Lysis of RBCs (mentioned above) subsequent to their continuous release from the clot during its clearance (33).

Glutamic acid, as an important EAA, is the most extensively studied amino acid in SAH. Levels of glutamic acid in the interstitial fluid increase within minutes of SAH, peak at 40 min, and remain elevated for days (49). The increase is most pronounced in patients with acute ischaemic neurological deficit (50). Increase in interstitial glutamic acid was identified as one of the earliest markers of impending ischaemia, typically increasing before the onset of clinical symptoms (27, 50). Our results are in line with these observations. Glutamic acid, aspartic acid, and 2-aminoadipic acid levels increase on days 0–3 post-SAH, and are all significantly higher at some point in the poor outcomes group of patients. In fact, in this group, outcome was most accurately predicted by 2-aminoadipic acid levels in CSF on day 10 post-SAH. 2-Aminoadipic acid is a structural homologue of glutamic acid and a natural product of lysine metabolism in mammalian cells (51). Huck et al. described gliotoxic properties of this amino acid (52), while Kato et al. observed enhanced susceptibility of glial cell to oxidative stress after 2-aminoadipic acid administration (53).

In this prospective observational study, some limitations need to be considered. First, specific enrolment criteria (acute HCP and EVD insertion) interfere with typical SAH pathophysiology. They will aggravate the SAH-associated brain injury and may well alter amino acid concentrations. Consequently, our observations may only be applicable to this subpopulation of SAH patients. Second, the relatively small number of examined samples and enrolled patients limits the extent of our conclusions. Nevertheless, the statistical relationships in our study follow the pattern of large scale studies (e.g., high correlation between admission status and treatment outcome). Third, EVD infection and CNS microbial inflammation could potentially affect the results, but our protocols specifically aim to minimise such problems. We have assumed a relationship between CSF and interstitial fluid, but this may itself be corrupted by the pathology. Future studies should include more good-grade patients without severe complications (e.g., acute HCP) with CSF drawn by LP.

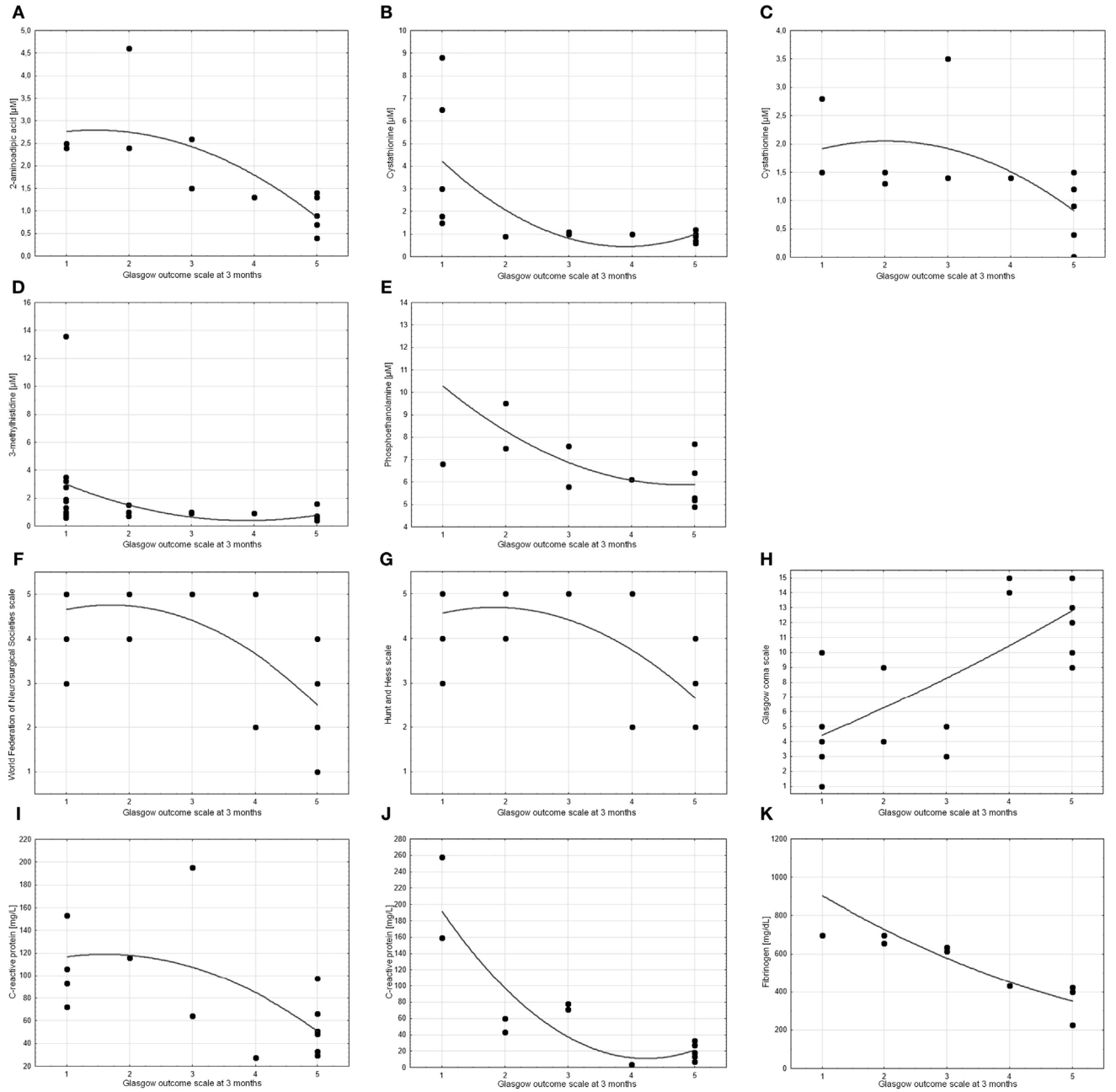


**FIGURE 4** | Scatter charts showing the correlation between treatment outcome (measured by Glasgow Outcome Scale at 3 months) and other parameters. Spearman's test revealed significant correlations (cc > 0.6 or cc < −0.6) for **(A)** 2-aminoadipic acid on day 10 post-SAH (cc = −0.81), **(B,C)** cystathionine on day 5 post-SAH (cc = −0.72) and day 10 post-SAH (cc = −0.67), **(D)** 3-methylhistidine on days 0–3 post-SAH (cc = −0.64), **(E)** *o*-phosphoethanolamine on day 10 post-SAH (cc = −0.62), **(F)** World Federation of Neurosurgical Societies scale (cc = −0.64), **(G)** Hunt and Hess scale (cc = −0.61), **(H)** Glasgow coma scale (cc = 0.72), **(I,J)** C-reactive protein on day 5 post-SAH (cc = −0.64) and day 10 post-SAH (cc = −0.79), and **(K)** fibrinogen on day 10 post-SAH (cc = −0.97). *p* values are <0.05 in all cases.

## CONCLUSION

Aneurysmal subarachnoid haemorrhage leads to a generalised increase of amino acids and related compounds in CSF. The patterns of concentrations differ between good and poor outcome patients. Increased EAAs are strongly indicative of poor outcome.

## ETHICS STATEMENT

This is a prospective observational study conducted in a single medical centre in accordance with the Declaration of Helsinki. The local bioethics committee approved the study protocol, consenting protocol, and consent forms.

## AUTHOR CONTRIBUTIONS

BS conceived and designed the study. BS, NW, RJa, and RJu analysed and interpreted the patient clinical data. BU, SP, AK, and ZK performed the amino acid assay and interpreted the laboratory data. BW performed statistical analysis. BS, NW, BW, and BU wrote the manuscript.

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CORRECTION
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# Corrigendum: Amino Acids in Cerebrospinal Fluid of Patients With Aneurysmal Subarachnoid Haemorrhage: An Observational Study

*Bartosz Sokół[1]*, Bartosz Urbaniak[2], Norbert Wąsik[1], Szymon Plewa[2], Agnieszka Klupczyńska[2], Roman Jankowski[1], Barbara Więckowska[3], Robert Juszkat[4] and Zenon Kokot[2]*

[1] Department of Neurosurgery, Poznan University of Medical Sciences, Poznan, Poland, [2] Faculty of Pharmacy, Department of Inorganic and Analytical Chemistry, Poznan University of Medical Sciences, Poznan, Poland, [3] Department of Computer Science and Statistics, Poznan University of Medical Sciences, Poznan, Poland, [4] Department of General and Interventional Radiology, Poznan University of Medical Sciences, Poznan, Poland

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****Correspondence:***
*Bartosz Sokół*
*bartosz.sokol@ump.edu.pl*

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**A corrigendum on**

**Amino Acids in Cerebrospinal Fluid of Patients with Aneurysmal Subarachnoid Haemorrhage: An Observational Study**

*by Sokół, B., Urbaniak, B., Wasik, N., Plewa, S., Klupczynska, A., Jankowski, R., et al. (2017). Front. Neurol. 8:438. doi: 10.3389/fneur.2017.00438*

In the original article, there were mistakes in **Table 2**, **Table 3**, **Table 4**, **Figure 1**, **Figure 2**, **Figure 3** and **Figure 4**.

In all mentioned tables and figures, quantity of amino acids was described in mM instead of μM. The corrected tables and figures appear below. The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way.

The original article has been updated.

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**TABLE 2 |** Differences in CSF amino acid level at day 0-3, 5, and 10 post-SAH in healthy individuals (control group) and SAH patients (study group).

| Amino acid | Control group | Study group, day 0-3 post-SAH | Control group vs. Study group, day 0-3 post-SAH. *P*-value | Study group, day 5 post-SAH | Control group vs. Study group, day 5 post-SAH. *P*-value | Study group, day 10 post-SAH | Control group vs. Study group, day 10 post-SAH. *P*-value |
|---|---|---|---|---|---|---|---|
| O-phosphoethanolamine (μM) | 3.00 | 5.95 | **<0.01** | 5.15 | **<0.001** | 6.60 | **<0.001** |
| Ethylamine (μM) | 9.80 | 9.40 | 0.798 | 9.65 | **<0.001** | 11.10 | 0.292 |
| Taurine (μM) | 7.00 | 16.00 | **<0.001** | 14.30 | **<0.001** | 10.75 | **<0.01** |
| Asparagine (μM) | 5.70 | 10.35 | **0.01** | 19.50 | **<0.001** | 21.75 | **<0.001** |
| Serine (μM) | 24.20 | 55.70 | **<0.001** | 75.40 | **<0.001** | 83.75 | **<0.001** |
| Glycine (μM) | 8.90 | 38.75 | **<0.001** | 34.85 | **<0.001** | 35.20 | **<0.001** |
| Hydroxyproline (μM) | 0.60 | 1.00 | 0.164 | 1.35 | 0.658 | 1.35 | 0.289 |
| Glutamine (μM) | 407.60 | 520.65 | **<0.01** | 791.10 | **<0.001** | 796.30 | **<0.001** |
| Aspartic acid (μM) | 0.50 | 1.55 | **<0.001** | 0.80 | 0.175 | 0.95 | 0.054 |
| Citruline (μM) | 1.60 | 2.75 | **0.011** | 2.55 | **<0.01** | 3.30 | **<0.01** |
| Threonine (μM) | 24.80 | 29.05 | 0.079 | 56.10 | **<0.001** | 63.40 | **<0.001** |
| Beta-Alanine (μM) | 20.30 | 22.00 | **0.040** | 29.00 | **<0.01** | 27.30 | **<0.01** |
| Alanine (μM) | 35.00 | 88.05 | **<0.001** | 114.55 | **<0.001** | 122.55 | **<0.001** |
| Glutamic acid (μM) | 1.20 | 5.40 | **<0.001** | 3.00 | **<0.001** | 2.35 | **<0.01** |
| Histidine (μM) | 11.40 | 28.20 | **<0.001** | 43.35 | **<0.001** | 40.20 | **<0.001** |
| 3-Methylhistidine (μM) | 0.50 | 1.00 | **<0.01** | 1.40 | **<0.001** | 1.05 | **<0.01** |
| 2-Aminoadipic acid (μM) | 0.00 | 1.65 | **<0.001** | 2.10 | **<0.001** | 1.45 | **<0.001** |
| Gamma-aminobutyric acid (μM) | 0.40 | 0.50 | 0.699 | 0.30 | **0.012** | 0.20 | 0.051 |
| 3-Aminoisobutyric acid (μM) | 0.50 | 0.30 | 0.668 | 0.30 | 0.563 | 0.35 | 0.806 |
| 2-Aminobutyric acid (μM) | 3.20 | 6.20 | **<0.01** | 6.60 | **<0.001** | 7.30 | **<0.001** |
| Arginine (μM) | 15.70 | 19.25 | 0.062 | 26.60 | **<0.001** | 24.35 | **<0.01** |
| Proline (μM) | 0.80 | 11.80 | **<0.001** | 13.95 | **<0.001** | 16.15 | **<0.001** |
| Ornithine (μM) | 4.30 | 19.80 | **<0.001** | 17.95 | **<0.001** | 17.75 | **<0.001** |
| Cystathionine (μM) | 0.30 | 2.05 | **<0.001** | 1.05 | **<0.001** | 1.40 | **<0.001** |
| Cysteine (μM) | 0.40 | 2.05 | **<0.001** | 1.75 | **<0.001** | 1.65 | **<0.001** |
| Lysine (μM) | 22.50 | 53.80 | **<0.001** | 73.05 | **<0.001** | 72.40 | **<0.001** |
| Methionine (μM) | 3.10 | 6.20 | **<0.01** | 11.70 | **<0.001** | 13.65 | **<0.001** |
| Valine (μM) | 16.20 | 38.65 | **<0.01** | 66.20 | **<0.001** | 76.30 | **<0.001** |
| Tyrosine (μM) | 7.80 | 26.70 | **<0.001** | 36.20 | **<0.001** | 37.30 | **<0.001** |
| Isoleucine (μM) | 4.40 | 9.65 | **0.001** | 13.05 | **<0.001** | 15.05 | **<0.001** |
| Leucine (μM) | 11.40 | 29.20 | **<0.001** | 43.50 | **<0.001** | 56.80 | **<0.001** |
| Phenylalanine (μM) | 9.40 | 26.45 | **<0.001** | 43.25 | **<0.001** | 44.45 | **<0.001** |
| Tryptophan (μM) | 1.90 | 9.70 | **<0.001** | 13.60 | **<0.001** | 12.85 | **<0.001** |

*Median levels of the amino acids are presented in the table. P-values were calculated either by Mann–Whitney or t-Student test. Amino acids increasing significantly during SAH (on day 0–3, 5, or 10) are shown in bold.*

**TABLE 3 |** Differences in monitored parameters at day 0-3, 5, and 10 post-SAH in patients with good (GO-SAH) and poor (PO-SAH) treatment outcome.

| Monitored parameter | Day 0–3 post-SAH | | | Day 5 post-SAH | | | Day 10 post-SAH | | |
|---|---|---|---|---|---|---|---|---|---|
| | GO-SAH | PO-SAH | GO-SAH vs. PO-SAH. *P*-value | GO-SAH | PO-SAH | GO-SAH vs. PO-SAH. *P*-value | GO-SAH | PO-SAH | GO-SAH vs. PO-SAH. *P*-value |
| C-reactive protein (mg/l) | 125.00 | 130.00 | 0.715 | 50.70 | 115.80 | **0.020** | 22.40 | 109.70 | 0.139 |
| White blood cell count ($10^6$/$mm^3$) | 9.86 | 17.47 | **<0.001** | 10.57 | 15.03 | **0.044** | 9.66 | 16.84 | 0.445 |
| Haemoglobin (g/dl) | 12.10 | 13.10 | 0.212 | 11.30 | 10.90 | 0.262 | 10.60 | 11.50 | 0.672 |
| Temperature ($^O$C) | 37.00 | 37.00 | 0.736 | 37.40 | 37.20 | 0.273 | 36.90 | 35.70 | 0.512 |
| Fibrinogen (mg/dl) | 485.00 | 550.00 | 0.879 | 509.00 | 676.00 | 0.257 | 428.00 | 698.00 | **0.014** |
| O-phosphoethanolamine (μM) | 4.10 | 6.80 | 0.161 | 4.90 | 7.70 | 0.161 | 5.95 | 8.50 | 0.019 |
| Taurine (μM) | 13.10 | 19.40 | **0.038** | 14.60 | 13.40 | 0.514 | 12.20 | 7.35 | 0.138 |
| Asparagine (μM) | 9.00 | 11.50 | 0.256 | 30.20 | 12.50 | 0.397 | 22.90 | 20.45 | 0.423 |
| Serine (μM) | 45.10 | 76.50 | 0.161 | 77.00 | 61.40 | 0.947 | 79.50 | 94.75 | 0.503 |
| Glycine (μM) | 29.60 | 41.00 | 0.182 | 30.00 | 43.00 | 0.204 | 35.20 | 35.65 | 0.671 |
| Hydroxyproline (μM) | 0.70 | 1.30 | 0.066 | 1.70 | 0.80 | 0.518 | 1.35 | 1.10 | 0.793 |
| Ethylaminev (μM) | 5.80 | 15.80 | 0.102 | 7.90 | 12.10 | 0.134 | 8.85 | 14.50 | 0.35 |
| Glutamine (μM) | 489.90 | 602.50 | 0.182 | 837.30 | 505.10 | 0.585 | 852.50 | 715.60 | 0.483 |
| Aspartic acid (μM) | 0.80 | 2.20 | **0.038** | 0.40 | 1.00 | 0.071 | 0.95 | 1.30 | 0.551 |
| Citruline (μM) | 1.90 | 5.90 | **0.035** | 2.50 | 3.10 | 0.396 | 3.30 | 3.30 | 1 |
| Threonine (μM) | 25.90 | 50.00 | 0.35 | 86.30 | 34.20 | 0.327 | 63.40 | 57.95 | 0.298 |
| Beta-Alanine (μM) | 28.30 | 22.00 | 0.07 | 30.00 | 28.60 | 0.497 | 26.85 | 27.85 | 0.893 |
| Alanine (μM) | 70.70 | 138.10 | 0.109 | 115.50 | 113.60 | 0.447 | 120.30 | 141.70 | 0.954 |
| Glutamic acid (μM) | (μM)3.40 | 9.30 | **0.038** | 2.70 | 5.80 | **0.024** | 2.00 | 17.10 | 0.269 |
| Histidine (μM) | 18.90 | 38.10 | 0.256 | 63.60 | 23.70 | 0.711 | 43.55 | 37.45 | 0.753 |
| 3-Methylhistidine (μM) | 0.70 | 1.50 | **<0.01** | 1.80 | 1.20 | 0.958 | 1.35 | 1.05 | 0.733 |
| 2-Aminoadipic acid (μM) | 1.30 | 1.70 | 0.076 | 1.50 | 4.10 | 0.071 | 1.30 | 2.45 | **0.033** |
| Gamma-aminobutyric acid (μM) | 0.30 | 0.90 | **0.043** | 0.30 | 0.30 | 0.932 | 0.35 | 0.15 | 0.266 |
| 3-Aminoisobutyric acid (μM) | 0.20 | 0.40 | 0.066 | 0.30 | 0.30 | 0.958 | 0.35 | 0.45 | 0.93 |
| 2-Aminobutyric acid | 4.60 | 8.20 | 0.088 | 11.40 | 5.50 | 0.542 | 10.75 | 6.50 | 0.124 |
| Arginine (μM) | 18.50 | 22.30 | 0.182 | 30.90 | 26.00 | 0.525 | 24.35 | 20.45 | 0.21 |
| Proline (μM) | 4.50 | 14.80 | 0.204 | 18.70 | 10.90 | 0.672 | 16.15 | 18.40 | 0.759 |
| Ornithine (μM) | 7.60 | 27.50 | **0.033** | 17.30 | 28.30 | 0.09 | 17.35 | 33.05 | 0.552 |
| Cystathionine (μM) | 0.80 | 2.80 | **<0.01** | 1.00 | 1.80 | 0.089 | 1.30 | 1.50 | 0.199 |
| Cysteine (μM) | 1.60 | 2.30 | 0.141 | 1.50 | 2.20 | 0.243 | 1.95 | 1.40 | 0.329 |
| Lysine (μM) | 43.00 | 74.50 | 0.095 | 87.10 | 55.90 | 0.341 | 72.40 | 72.60 | 0.612 |
| Methionine (μM) | 4.60 | 12.30 | 0.204 | 16.20 | 7.70 | 0.491 | 13.65 | 13.70 | 0.687 |
| Valine (μM) | 31.80 | 62.40 | 0.109 | 102.50 | 33.60 | 0.397 | 87.75 | 72.20 | 0.377 |
| Tyrosine (μM) | 23.00 | 32.50 | 0.083 | 36.60 | 35.80 | 0.876 | 38.60 | 37.30 | 0.676 |
| Isoleucine (μM) | 6.00 | 13.00 | **0.045** | 17.60 | 11.80 | 0.597 | 15.05 | 13.70 | 0.45 |
| Leucine (μM) | 22.90 | 39.70 | 0.109 | 80.30 | 29.80 | 0.397 | 60.95 | 50.55 | 0.532 |
| Phenylalanine (μM) | 21.20 | 38.80 | 0.062 | 51.30 | 32.40 | 0.665 | 45.40 | 44.45 | 0.913 |
| Tryptophan (μM) | 8.00 | 12.20 | 0.066 | 13.60 | 13.60 | 0.606 | 12.60 | 13.55 | 0.804 |

*Median levels of the amino acids are presented in the table. P-values were calculated either by Mann–Whitney or t-Student test. Significant differences are shown in bold.*

**TABLE 4 |** Amino acid level changes in time in good outcome SAH patients with (GO-SAH).

| Parameter | Day 0–3 post-SAH | Day 5 post-SAH | Day 10 post-SAH | Omnibus test *P*-value | Day 0–3 vs. Day 5 *P*-value *Post-hoc* | Day 0–3 vs. Day 10 *P*-value *Post-hoc* | Day 5 vs. Day 10 *P*-value *Post-hoc* |
|---|---|---|---|---|---|---|---|
| C-reactive protein (mg/l) | 125.00 | 50.70 | 27.00 | **0.049** | 0.345 | **0.012** | 0.073 |
| White blood cell count ($10^6$/$mm^3$) | 9.86 | 10.57 | 9.34 | 0.631 | | | |
| Haemoglobin (g/dl) | 12.10 | 10.60 | 10.50 | 0.066 | | | |
| Temperature ($^O$C) | 37.00 | 37.30 | 36.90 | 0.382 | | | |
| Fibrinogen (mg/dl) | 485.00 | 442.00 | 423.00 | 0.327 | | | |
| O-phosphoethanolamine (μM) | 6.20 | 5.00 | 5.80 | 0.325 | | | |
| Taurine (μM) | 13.90 | 14.60 | 10.80 | 0.867 | | | |
| Asparagine (μM) | 8.40 | 18.30 | 21.20 | 0.180 | | | |
| Serine (μM) | 45.10 | 73.80 | 76.10 | **0.031** | **0.039** | **0.013** | 0.566 |
| Glycine (μM) | 29.60 | 32.60 | 42.50 | 0.565 | | | |
| Hydroxyproline (μM) | 0.70 | 1.10 | 1.40 | 0.094 | | | |
| Ethylaminev (μM) | 5.70 | 10.10 | 9.80 | **0.039** | 0.078 | **<0.01** | 0.187 |
| Glutamine (μM) | 454.30 | 818.70 | 828.30 | **0.001** | **<0.01** | **<0.001** | 0.565 |
| Aspartic acid (μM) | 0.70 | 0.40 | 1.20 | 0.215 | | | |
| Citruline (μM) | 1.90 | 2.40 | 2.80 | 0.069 | | | |
| Threonine (μM) | 22.90 | 52.50 | 61.20 | **0.012** | 0.128 | **<0.01** | **<0.01** |
| Beta-Alanine (μM) | 28.30 | 26.80 | 26.70 | 0.553 | | | |
| Alanine (μM) | 70.70 | 106.20 | 120.30 | 0.124 | | | |
| Glutamic acid (μM) | 4.50 | 2.70 | 2.10 | 0.898 | | | |
| Histidine (μM) | 16.80 | 38.10 | 37.40 | 0.156 | | | |
| 3-Methylhistidine (μM) | 0.60 | 1.50 | 0.90 | 0.369 | | | |
| 2-Aminoadipic acid (μM) | 1.30 | 1.50 | 1.30 | 0.129 | | | |
| Gamma-aminobutyric acid (μM) | 0.30 | 0.20 | 0.50 | 0.633 | | | |
| 3-Aminoisobutyric acid (μM) | 0.20 | 0.30 | 0.30 | 0.215 | | | |
| 2-Aminobutyric acid (μM) | 4.60 | 11.40 | 11.40 | **0.010** | **0.019** | **<0.01** | 0.427 |
| Arginine (μM) | 18.50 | 26.50 | 24.10 | **0.050** | 0.073 | **0.012** | 0.345 |
| Proline (μM) | 3.40 | 11.90 | 15.20 | 0.062 | | | |
| Ornithine (μM) | 7.60 | 17.30 | 14.30 | 0.368 | | | |
| Cystathionine (μM) | 1.00 | 1.00 | 1.20 | 0.648 | | | |
| Cysteine (μM) | 1.60 | 1.70 | 2.10 | 0.368 | | | |
| Lysine (μM) | 43.00 | 83.20 | 70.80 | **0.017** | **0.017** | **<0.01** | 0.735 |
| Methionine (μM) | 4.60 | 10.00 | 11.90 | **0.008** | **0.029** | **<0.01** | 0.200 |
| Valine (μM) | 28.20 | 61.60 | 78.10 | **0.015** | **0.017** | **<0.01** | 0.683 |
| Tyrosine (μM) | 23.00 | 35.10 | 28.50 | **0.020** | **0.023** | **<0.01** | 0.647 |
| Isoleucine (μM) | 5.90 | 11.10 | 13.60 | **0.024** | 0.081 | **<0.01** | 0.219 |
| Leucine (μM) | 22.60 | 39.70 | 60.50 | **0.024** | **0.022** | **0.013** | 0.801 |
| Phenylalanine (μM) | 20.90 | 35.20 | 37.50 | **0.038** | **0.040** | **0.018** | 0.700 |
| Tryptophan (μM) | 8.00 | 11.80 | 11.10 | 0.072 | | | |

*Median levels of the amino acids are presented in the table. Omnibus statistical test (Friedman or repeated measures ANOVA) revealed significant changes in concentration of 13 amino acids (shown in bold). Next, post-hoc analysis (Conover–Iman for Friedman test and Fisher for repeated measures ANOVA) was run for those 13 amino acids to specify between what time points (day 0–3, 5, or 10 post-SAH) the differences in concentration were significant (shown in bold).*

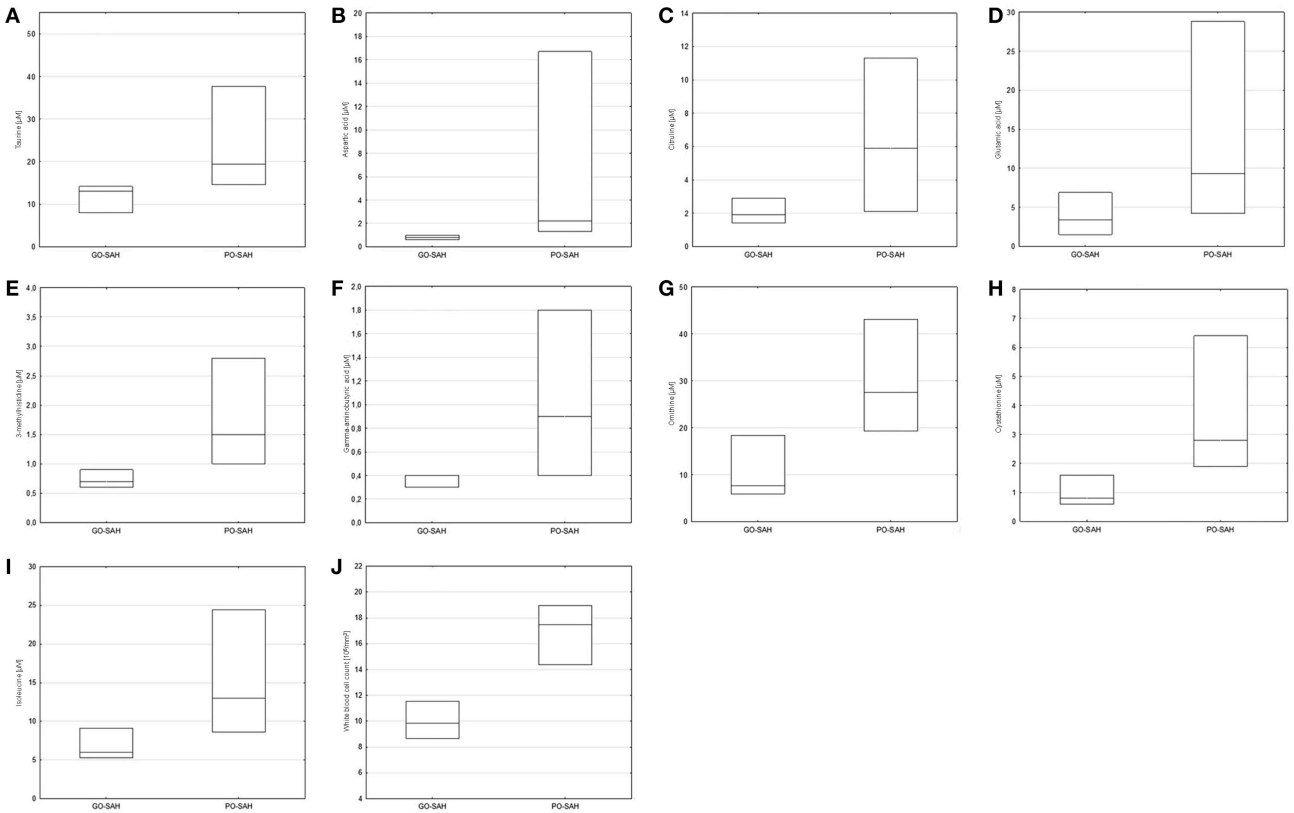


**FIGURE 1 |** Significant differences in monitored parameters on day 0–3 post-SAH between patients with good (GO-SAH) and poor (PO-SAH) treatment outcome. Mann–Whitney test revealed significantly higher levels of: **(A)** taurine ($p = 0.038$), **(B)** aspartic acid ($p = 0.038$), **(C)** citruline ($p = 0.035$), **(D)** glutamic acid ($p = 0.038$), **(E)** 3-methylhistidine ($p < 0.01$), **(F)** gamma-aminobutyric acid ($p = 0.043$), **(G)** ornithine ($p = 0.033$), **(H)** cystathionine ($p < 0.01$), **(I)** isoleucine ($p = 0.045$) in patients with poor outcome. *T*-Student test revealed significantly higher level of white blood cell count ($p < 0.01$) in patients with poor outcome **(J)**. In all cases median levels and the 25th and 75th percentile are presented.

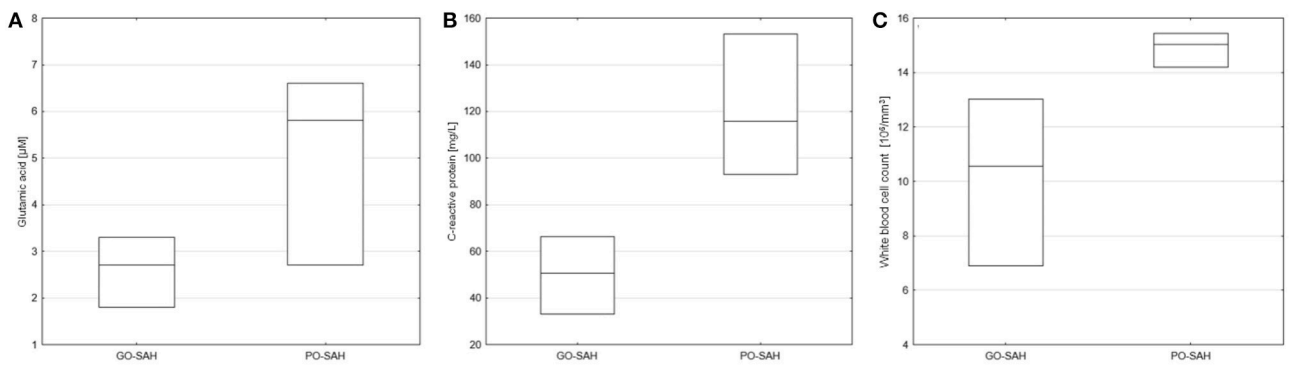


**FIGURE 2 |** Significant differences in monitored parameters on day 5 post-SAH between patients with good (GO-SAH) and poor (PO-SAH) treatment outcome. **(A)** *T*-Student test revealed significantly higher levels of glutamic acid ($p = 0.041$) in patients with poor outcome. Mann–Whitney test revealed significantly higher C-reactive protein level ($p = 0.020$) **(B)** and white blood cell count ($p = 0.044$) **(C)** in patients with poor outcome. In all cases median levels and the 25th and 75th percentile are presented.

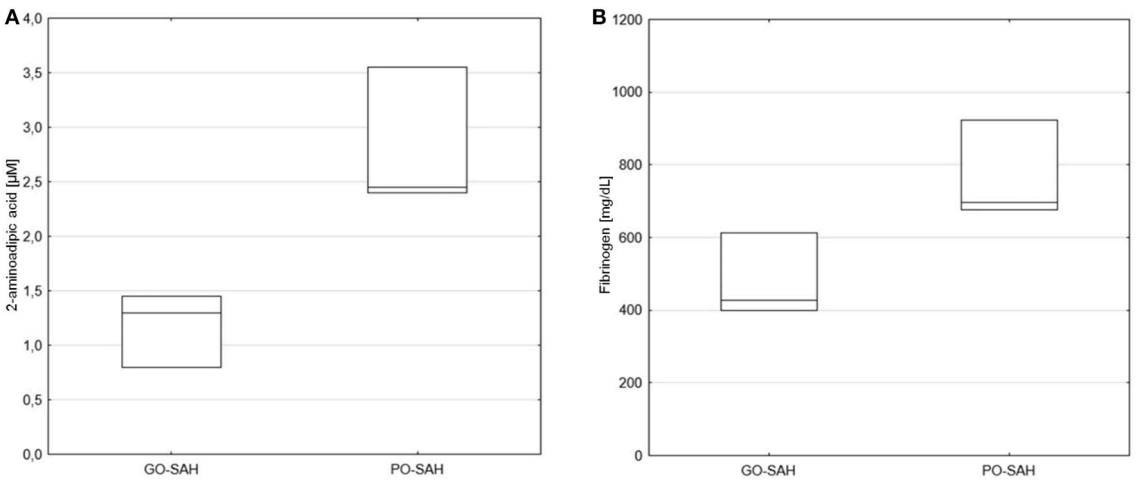


**FIGURE 3 |** Significant differences in monitored parameters on day 10 post-SAH between patients with good (GO-SAH) and poor (PO-SAH) treatment outcome. Mann–Whitney test revealed significantly higher 2-aminoadipic acid ($p = 0.033$) **(A)** and fibrinogen ($p = 0.014$) **(B)** levels in patients with poor outcome. Median levels and the 25th and 75th percentile are presented.

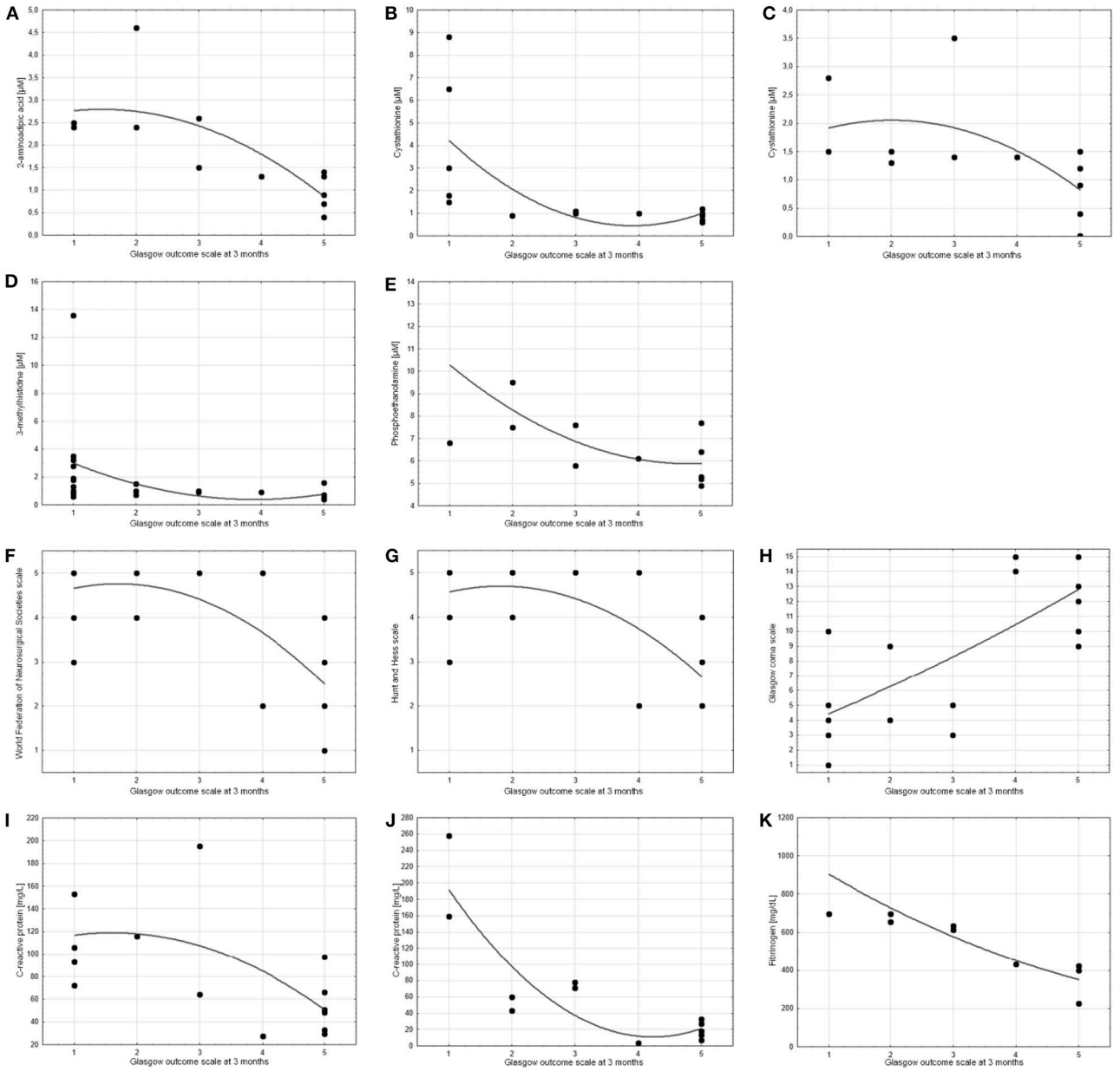


**FIGURE 4 |** Scatter charts showing the correlation between treatment outcome (measured by Glasgow outcome scale at 3 months) and other parameters**.** Spearman's test revealed significant correlations (cc > 0.6 or cc < −0.6) for: **(A)** 2-aminoadipic acid on day 10 post-SAH (cc = −0.81), **(B,C)** cystathionine on day 5 post-SAH (cc = −0.72) and day 10 post-SAH (cc = −0.67), **(D)** 3-methylhistidine on day 0–3 post-SAH (cc = −0.64), **(E)** o-phosphoethanolamine on day 10 post-SAH (cc = −0.62), **(F)** World Federation of Neurosurgical Societies scale (cc = −0.64), **(G)** Hunt and Hess scale (cc = −0.61), **(H)** Glasgow coma scale (cc = 0.72), **(I,J)** C-reactive protein on day 5 post-SAH (cc = −0.64) and day 10 post-SAH (cc = −0.79), **(K)** Fibrinogen on day 10 post-SAH (cc = −0.97). *P*-values are <0.05 in all cases.

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# Xenon Reduces Neuronal Hippocampal Damage and Alters the Pattern of Microglial Activation after Experimental Subarachnoid Hemorrhage: A Randomized Controlled Animal Trial

*Michael Veldeman[1,2]*, Mark Coburn[2], Rolf Rossaint[2], Hans Clusmann[1], Kay Nolte[3], Benedikt Kremer[1] and Anke Höllig[1]*

[1] Department of Neurosurgery, RWTH Aachen University Hospital, Aachen, Germany, [2] Department of Anesthesiology, RWTH Aachen University Hospital, Aachen, Germany, [3] Department of Neuropathology, RWTH Aachen University Hospital, Aachen, Germany

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*J. Marc Simard, University of Maryland, Baltimore, United States*

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***Correspondence:**
*Michael Veldeman*
*mveldeman@ukaachen.de*

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**Objective:** The neuroprotective properties of the noble gas xenon have already been demonstrated using a variety of injury models. Here, we examine for the first time xenon's possible effect in attenuating early brain injury (EBI) and its influence on posthemorrhagic microglial neuroinflammation in an *in vivo* rat model of subarachnoid hemorrhage (SAH).

**Methods:** Sprague-Dawley rats ($n = 22$) were randomly assigned to receive either Sham surgery ($n = 9$; divided into two groups) or SAH induction *via* endovascular perforation ($n = 13$, divided into two groups). Of those randomized for SAH, 7 animals were post-operatively ventilated with 50 vol% oxygen/50 vol% xenon for 1 h and 6 received 50 vol% oxygen/50 vol% nitrogen (control). The animals were sacrificed 24 h after SAH. Of each animal, a cerebral coronal section (–3.60 mm from bregma) was selected for assessment of histological damage 24 h after SAH. A 5-point neurohistopathological severity score was applied to assess neuronal cell damage in H&E and NeuN stained sections in a total of four predefined anatomical regions of interest. Microglial activation was evaluated by a software-assisted cell count of Iba-1 stained slices in three cortical regions of interest.

**Results:** A diffuse cellular damage was apparent in all regions of the ipsilateral hippocampus 24 h after SAH. Xenon-treated animals presented with a milder damage after SAH. This effect was found to be particularly pronounced in the medial regions of the hippocampus, CA3 ($p = 0.040$), and dentate gyrus (DG $p = 0.040$). However, for the CA1 and CA2 regions, there were no statistical differences in neuronal damage according to our histological scoring. A cell count of activated microglia was lower in the cortex of xenon-treated animals. This difference was especially apparent in the left piriform cortex ($p = 0.017$).

**Abbreviations:** SAH, subarachnoid hemorrhage; ICP, intracranial pressure; rCBF, regional cerebral blood flow; ROI, region of interest; ICA, internal carotid artery; HPF, high power field.

**Conclusion:** In animals treated with 50 vol% xenon (for 1 h) after SAH, a less pronounced neuronal damage was observed for the ipsilateral hippocampal regions CA3 and DG, when compared to the control group. In xenon-treated animals, a lower microglial cell count was observed suggesting an immunomodulatory effect generated by xenon. As for now, these results cannot be generalized as only some hippocampal regions are affected. Future studies should assess the time and localization dependency of xenon's beneficial properties after SAH.

**Keywords: subarachnoid hemorrhage, early brain injury, animal model, xenon, neuroprotection**

# INTRODUCTION

## Background

Aneurysmal subarachnoid hemorrhage (SAH) is a subtype of stroke occurring at a relative young age causing either death or disability in many patients (1). Few people recover without impairments (2). The annual incidence is estimated to be about 9.1 patients per 100,000 (3). In around 85% of spontaneous SAHs, the underlying cause is the rupture of a cerebral aneurysm (4). The resulting increase in intracranial pressure (ICP), disruption of the blood–brain barrier and global ischemia contribute to early brain injury (EBI) (5). These injuries within the first 72 h of the initial ictus account for the later development of vasospasm and delayed cerebral ischemia (6). There is a substantial interest in EBI and in ways to reduce initial cerebral damage and thus indirectly attenuate secondary injuries.

The neuroprotective effect of the noble gas xenon has been well established in animal experiments for focal and global cerebral ischemia (7–11). Xenon has a proven additive neuroprotective effect to hypothermia in models of neonatal asphyxia (12–14). So far, xenon treatment has not been examined in the context of SAH. Multiple animal models of SAH have been established. The two most commonly used are the cisternal injection model and the endovascular perforation model (15). We opted for the latter as it better mimics the pathophysiology of an aneurysm rupture, and it is probably more suitable to investigate EBI (16). In this trial, the neuroprotective properties of the noble gas xenon were examined in the early phase after SAH using an endovascular perforation rat model. Despite its cost, xenon has demonstrated minimal side effects in extensive anesthesia studies, making it an interesting future treatment in human trials aiming for neuroprotection (17–21). Furthermore, xenon has already been approved in Europe for use as a general anesthetic.

# MATERIALS AND METHODS

## Study Design

We performed a randomized four group controlled animal trial examining the neuroprotective effects of xenon inhalation (50 vol% for 1 h) with treatment initiation 1 h after SAH induction.

## Ethical Statement

The study protocol was approved by the government agency for animal use and protection (Protocol number: TVA 10416G1 approved by "Landesamt für Natur, Umwelt und Verbraucherschutz NRW," Recklinghausen, Germany), all experiments were conducted in accordance with the Guide for Care and Use of Laboratory Animals (National Research Council, and the Committee for the Update of the Guide for the Care and Use of laboratory Animals; 8th edition 2011).

## Animals

Male Sprague-Dawley rats (body weight 300–400 g, Charles River, Sulzfeld, Germany) were housed for at least 1 week before surgery with free access to food in a specific pathogen-free environment maintaining a 12-h light/dark cycle. Prior to anesthesia induction, animals were randomly assigned to one of the following four groups by lot drawing: Sham $N_2$ (Sham surgery after 1 h delay followed by ventilation with 50 vol% $O_2$/50 vol% $N_2$ for 1 h), Sham Xe (Sham surgery after 1 h delay followed by ventilation with 50 vol% $O_2$/50 vol% Xe for 1 h), SAH $N_2$ (SAH induction after 1 h delay followed by ventilation with 50 vol% $O_2$/50 vol% $N_2$ for 1 h), and SAH Xe (SAH induction after 1 h delay followed by ventilation with 50 vol% $O_2$/50 vol% Xe for 1 h) (see **Figure 1**).

## Experimental Procedure

Anesthesia was induced by intraperitoneal injection of a mixture of midazolam (2 mg/kg), medetomidine (0.15 mg/kg), and

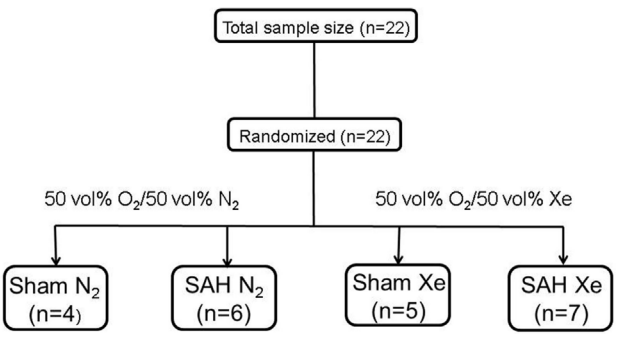


**FIGURE 1** | Flow chart of included animals. Animals were randomized by lot drawing in one of four groups. Sham $N_2$ (Sham surgery after 1 h delay followed by ventilation with 50 vol% $O_2$/50 vol% $N_2$ for 1 h), Sham Xe (Sham surgery after 1 h delay followed by ventilation with 50 vol% $O_2$/50 vol% Xe for 1 h), SAH $N_2$ (SAH induction after 1 h delay followed by ventilation with 50 vol% $O_2$/50 vol% $N_2$ for 1 h), and SAH Xe (SAH induction after 1 h delay followed by ventilation with 50 vol% $O_2$/50 vol% Xe for 1 h).

fentanyl (0.0075 mg/kg) (22, 23). A quarter of the initial dosage was injected in 30–45 min intervals to maintain anesthesia. Postoperative analgesia was started directly after surgery *via* intramuscular injection of metamizole (20 mg/kg) and continued until euthanasia (24 h after SAH induction). Animals were intubated using an 18 gauge i.v. cannula. Blood pressure was monitored by cannulation of the tail artery, electrolytes, and blood gases were monitored by repeated arterial blood gas analysis and body temperature was maintained at 37°C via a heating pad (Physitemp Instruments, Inc., Clifton, NJ, USA). After anesthesia induction, two laser Doppler flowmetry probes were fixated in proximity of the bregma to measure regional cerebral blood flow (rCBF), as previously described (Moor Instruments, Axminster, Devon, UK) (23). A left side parietal ICP probe was inserted for continuous ICP monitoring (Microsensor/Codman ICP Express Monitor, Codman/De Puy, Raynham, MA, USA). Baseline recordings of blood pressure, bilateral rCBF, heart rate, and ICP were done prior to surgery, during intervention, and 90 min thereafter (PowerLab, ADInstruments, Spechbach, Germany). SAH was induced by the polypropylene monofilament perforation technique initially described by Bederson et al. and modified by Veelken et al. (24, 25). The procedure was performed as previously described (26). After exposing the left common carotid artery, the left internal carotid artery (ICA) was identified and a 3–0 polypropylene suture with a diameter ranging from 200 to 250 µm (Prolene suture, Ethicon Inc., Somerville, NJ, USA) was advanced intravascularly. Perforation of the vessel and subsequent SAH was verified by a sudden increase in ICP and a bilateral decrease in rCBF. Sham-operated animals underwent the same anesthesia and surgical procedure, but the monofilament was advanced into the ICA without perforation of the vessel.

One hour after SAH induction or Sham surgery, the animals were ventilated for 1 h with either a mixture of 50 vol% $O_2$/50 vol% xenon (Air Liquide, Paris, France) or 50 vol% $O_2$/50 vol% $N_2$ (control group). After treatment, anesthesia was stopped and animals were allowed to recover spontaneously. Analgesic treatment with metamizole (20 mg/kg intramuscular application every 8 h) was carried on until euthanasia (24 h after SAH induction). Euthanasia was performed 24 h after SAH induction by exsanguination under deep anesthesia followed by decapitation. Brains were harvested and cut into 2 mm coronal slices, fixated in paraformaldehyde, and embedded in paraffin.

## Histology/Immunohistochemistry

Sections of 2 µm thickness were cut from the paraffin-embedded brain slices and placed on silane-coated slides. Of every animal, the same section 3.60 mm posterior to the bregma was searched based on anatomical landmarks. After deparaffinization, a section was routinely hematoxylin/eosin (H&E) stained. Two consecutive sections were de-waxed, rehydrated, and heated in citrate buffer for antigen retrieval. After blocking of non-specific binding by incubation in PBS containing 2% normal goat serum, one slide per animal was incubated for 1 h with anti-NeuN (Millipore, MA, USA) as primary antibody diluted in blocking solution and one slide with anti-Iba-1. Appropriate biotinylated secondary antibodies were used (1:200, Vector Laboratories Ltd., Peterborough, UK) for 15 min, followed by DAB visualization (DAKO, Carpinteria, CA, USA). Appropriate negative controls without the primary antibodies were performed.

## Neuronal Cell Damage

Neuronal cell damage was measured and quantified in four regions of the left hippocampus, in H&E and NeuN-stained section: CA1, CA2, CA3, and dentate gyrus (DG). A single high power field (HPF) was focused on the center of each of these four region of interest (ROI) and the image was photographed with an Axioplan microscope (ZEISS, Germany). An absolute neuronal cell count as well as a cell count of all ischemic damaged neurons was done using ImageJ/Fiji v 1.50 (ImageJ Software downloaded at https://imagej.nih.gov/ij). See **Figure 2**. Neuronal cell damage was cytomorphologically defined as a combination of hypereosinophilia, shrunken cytoplasm, and pyknotic nuclei. This counting process was done twice by a single investigator blinded to treatment allocation on two different time-points and results were compared for incongruence. In case of incongruence or doubt, the consecutive NeuN stained slices was consulted and the process was repeated. The ratio of damaged neurons too the complete neuronal cell count was graded into five categories (1 = 0–20%, 2 = 20–40%, 3 = 40–60%, 4 = 60–80%, and 5 = 80–100%). See **Figure 3**. The results of this scale for each ROI in the left hemisphere were then summed to yield an overall neurohistopathological severity score per animal.

## Microglial Activation

An absolute microglial cell count was performed in a similar fashion in the Iba-1 (ionized calcium-binding adapter molecule 1) stained sections. Three cortical regions of interest per animal were photographed. The absolute number of activated Iba-1-positive cells was software-assisted counted out in the

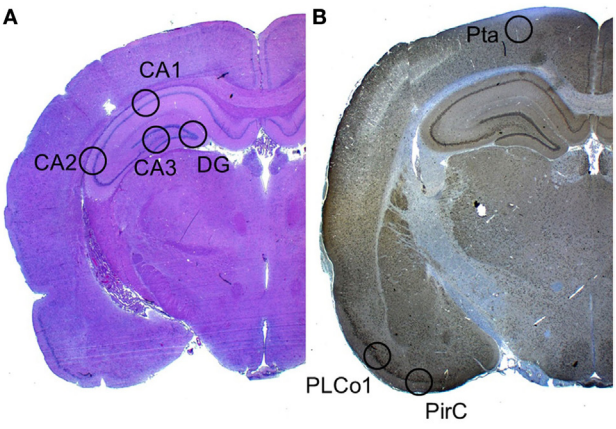


**FIGURE 2** | Selected regions of interest. **(A)** In H&E staining, a high power field was focused on four regions of interest: CA1, CA2, CA3, and DG. **(B)** In Iba-1 staining (the image displays a NeuN staining), a high power field was focused on the three cortical regions of interest: Pta, PLCo1, and Pir1.

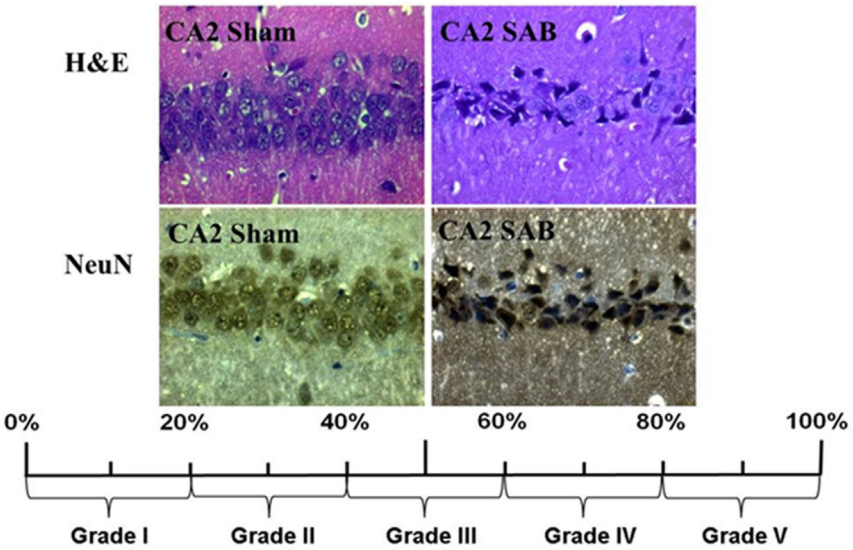


**FIGURE 3** | Histopathological severity score. Neuronal cell damage was evaluated in H&E and NeuN staining. Damaged neurons, characterized by hypereosinophilia, shrunken cytoplasm, and pyknotic nuclei, were software assisted counted and expressed as a ration to the total cell count per region. The resulting percentage was converted into grade 1 to grade 5.

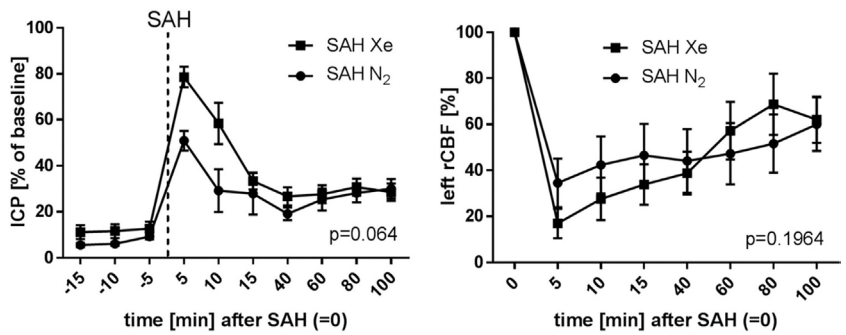


**FIGURE 4** | Course of ICP and rCBF. Graphs for intracranial pressure (ICP) and left regional cerebral blood flow (rCBF) are presented as mean ± SD of percentages of baseline values; time = 0 min is defined as subarachnoid hemorrhage (SAH). The courses did not differ between the xenon and control group (repeated measures analysis of variance).

lateral primary somatosensory cortex (Pta), postero-lateral cortex (PLCo1), and the piriform cortex (Pir1) of both hemispheres. **Figure 2** offers an overview of the selected regions of interest.

## Experimental Outcomes

The primary outcome was left side histopathological hippocampal damage as measured by our neurohistopathological severity score. The secondary outcome was microglial activation. In the initial trial design, a 24-h clinical evaluation using an 18- and 28-point scoring system was included. After the negative results in a similar study with argon, where we observed no difference in neurologic function in the acute phase (26), no short-term neurological evaluation was done in this trial.

## Statistical Methods

To estimate sample size data from previous experiments using argon as a neuroprotective agent were extrapolated (26). Using these data, an effect size of 0.62 was calculated (alpha: 0.1; beta: 0.8). As xenon is known to be more potent, we estimated an effect size of 0.7 (alpha: 0.1; beta: 0.8) resulting in a sample size of $n = 6$ (G*Power 3.9.1.2 downloaded at http://www.gpower.hhu.de/).

All statistical analyses were performed using SPSS v 23.0 (SPSS Inc., Chicago, IL, USA). All graphics were plotted using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). A $p$-value of $<0.05$ was considered statistically significant. After normality testing (Kolmogorov–Smirnov test), an unpaired $t$-test was used to analyze normally distributed numeric variables. Group comparisons were performed using

one-way or two-way ANOVA testing followed by the appropriate *post hoc* test. All data are presented as means ± SD unless stated otherwise.

# RESULTS

## Baseline Data

The course of ICP and left side rCBF is illustrated in **Figure 4**. The SAH Xe group reached an overall higher peak in ICP increase after SAH induction compared to the control group. However, this difference was not statistically significant ($p = 0.064$). The initial decrease in rCBF after SAH induction did not differ significantly between the xenon and control group ($p = 0.1964$).

## Neuronal Cell Damage in H&E and NeuN-Stained Sections

Routine hematoxylin and eosin staining was used to cytomorphologically quantify ischemic damage. Apoptotic neurons were clearly demarcated in the cell layers of all four assessed hippocampal regions. To compare treatment groups, the four scores (0–5) for each hippocampus were summed up to obtain a single score per animal between 0 and 20. Sham animals presented with mild baseline cell damage in all four hippocampal regions. The Sham $N_2$ group had a summed-up score of 4.5 ± 1.83. The Sham Xe group presented with a summed-up score of 10 ± 4.05. The SAH $N_2$ group had a summed-up score of 16 ± 2.52, and the SAH Xe group showed a summed-up score of 12.71 ± 4.13. These data are presented in **Figure 5**. The xenon-treated SAH group scored a lower summed-up score compared to the SAH $N_2$ group, suggesting a protective effect of xenon after SAH. These differences proved, however, not to be statistically significant ($p = 0.287$). There was a significant difference between the Sham $N_2$ and SAH $N_2$ groups ($p = 0.0001$) illustrating the overall damage caused by the induced SAH. For the summed-up scoring, there was no significant difference between the Sham Xe and SAH Xe groups ($p = 0.326$). Once comparing individual hippocampal regions, it became apparent that the more medial located CA3 and DG regions were generally more intensely damaged compared to the more lateral located CA1 and CA2 regions. In those regions with more pronounced damaged the inter-group differences

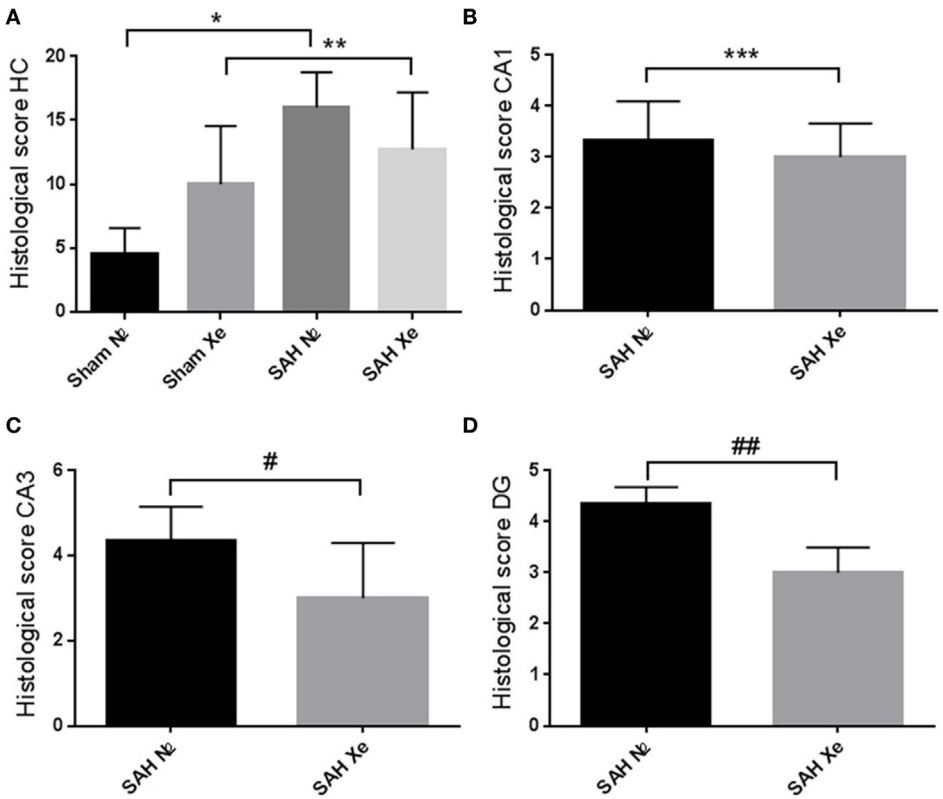


**FIGURE 5** | Hippocampal neuronal cell loss. Cytomorphological quantification of ischemic damage. (A) After summing up the four scores (0–5) per hippocampal region, a single score per animal between 0 and 20 was yielded. The summed-up scores were 4.5 ± 1.83 (Sham $N_2$), 10 ± 4.05 (Sham Xe), 16 ± 2.52 (SAH $N_2$), and 12.71 ± 4.13 (SAH Xe) group. There was no difference between the SAH $N_2$ and SAH Xe groups. There was a significant difference between the Sham $N_2$ and SAH $N_2$ groups, *p = 0.0001. There was no significant difference between Sham Xe and SAH $N_2$ groups, **p = 0.326. (B) The CA1 region of the SAH $N_2$ group and the SAH Xe group had a mean histopathological severity score of 3.33 ± 1.70 vs. 3.0 ± 1.60, ***p = 0.4280. (C) In the CA3 regions, a significant difference group-score was seen, 4.5 ± 0.76 (SAH $N_2$) vs. 2.71 ± 1.58 (SAH Xe) group, #p = 0.040. (D) The difference was also statistically significant in the DG regions 4.24 ± 1.38 vs. 3.09 ± 1.28, ##p = 0.040.

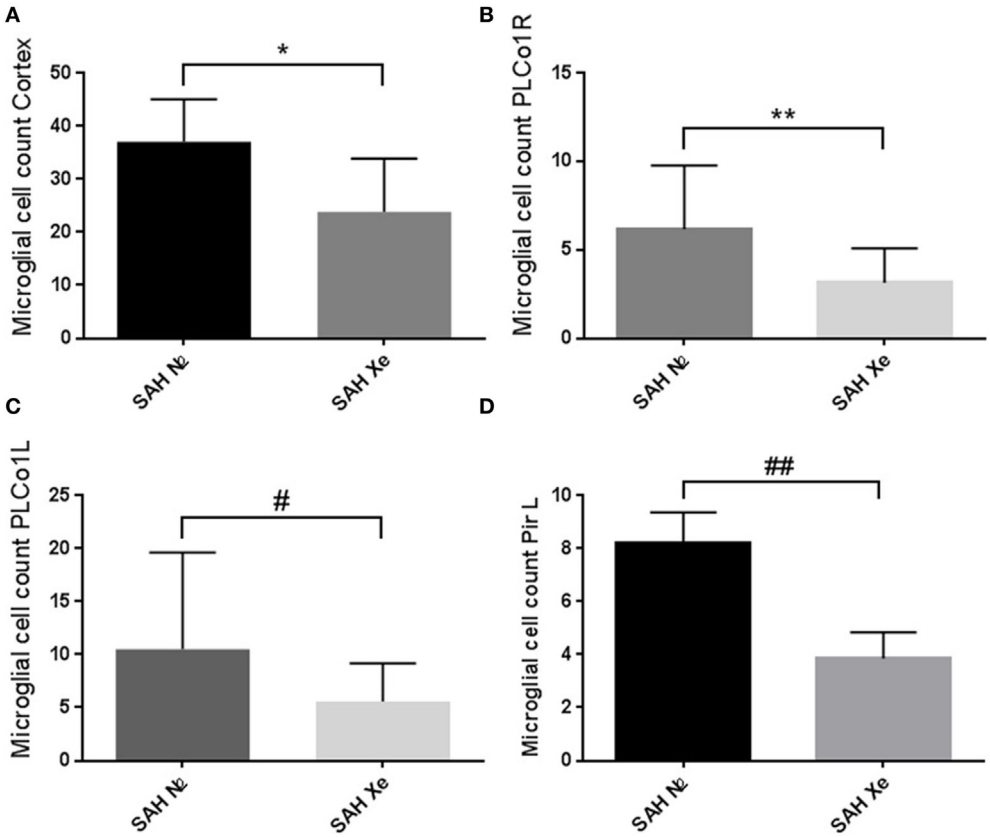


**FIGURE 6** | Cortical microglial cell count. Software assisted counting of Iba-1 positive cells in four cortical regions. **(A)** Comparing the summed up absolute cell count per animal, the xenon-treated animals showed a lower overall microglial cell count compared to the control groups, *$p$ = 0.1206. **(B,C)** Regions closer to the bleeding source showed a higher number of activated microglia (highest damage in the PLCo1L region) with an increase in the difference between treatment groups without statistical significance, **$p$ = 0.1680, #$p$ = 0.1002. **(D)** The difference in cell count was statistically significant in the left side piriform cortex (pir1 L). 8 ±1.16 microglial cells were counted in the control group vs. 4 ± 1.07 in the xenon group, ##$p$ = 0.017.

increased. Comparing the CA1 region of the SAH $N_2$ group and the SAH Xe group, the mean histopathological severity score was 3.33 ± 1.70 vs. 3.0 ± 1.60 ($p = 0.428$). Whereas comparing the CA3 regions in the SAH $N_2$ group, a mean score was yielded of 4.5 ± 0.76 vs. 2.71 ± 1.58 in the SAH Xe group ($p = 0.040$). The difference between the SAH $N_2$ and SAH Xe group also became statistically significant in the DG regions 4.24 ± 1.38 vs. 3.09 ± 1.28 ($p = 0.040$).

## Microglial Activation in Iba-1 Stained Sections

In the brain, ionized calcium-binding adapter molecule 1 (Iba-1) is specifically expressed in microglia and, therefore, can be used as a robust biomarker for these cell types. Activated microglial cells are morphologically characterized by cellular branches and are easily identified using confocal microscopy. We determined the presence of activated microglia within three cortical regions for each hemisphere. Two regions are located at the base of the brain, close to the source of the induced hemorrhage (PLCo1 and Pir1). One region was chosen more distal to the bleeding source in the cranial parietal cortex (Pta). Iba-1-positive cells were software-assisted quantified in a HPF, focusing on the center of each ROI. In comparison to Sham-operated rats, SAH animals showed a clear increase in Iba-1-positive cells in all three cortical regions, reflecting the early inflammatory response after SAH. Once the absolute cell count of all three regions was summed up per animal, it became apparent that xenon-treated animals showed a lower overall microglial cell count compared to the control groups. See **Figure 6**. Also in all SAH animals, those regions closer to the initial bleeding source (left side Pir1 and PLCo1) showed a higher number of activated microglia reflecting the spatial distribution of severity of neuronal damage around the area of primary hit. This effect has been previously described in a rat SAH perforation model (27). In those regions of greater damage and microglial activation, the number of microglial cells was lower in the xenon-treated animals. This difference was statistically significant in the left side pir1 L where an average of 8 ± 1.16 microglial cells were counted per HPF in the control group vs. 4 ± 1.07 cells in the xenon group, $p = 0.017$.

# DISCUSSION

We investigated the effects of xenon to attenuate EBI in a rat SAH model. Although a xenon-mediated protective effect was seen

in all hippocampal regions, the protective effect of xenon was enhanced in the CA3 and DG regions. Second, we have shown a reduction in cortical microglial activity in xenon-treated animals. An intraparenchymal accumulation of microglia cells was more pronounced in regions closer to the site of vessel perforation. This effect has been previously demonstrated in animal as well as human tissue samples (28). In this trial by Schneider et al., a centrifugal spreading of microglia accumulation developed over time, from the base of the cortex of both hemispheres, resembling a wave of intracerebral immune cell activation. Similarly, we saw a gradual decrease of microglial activation in regions farther away from the site of primary hit. In the base of the left hemisphere, the regions with the highest accumulation of inflammatory cells, the immunomodulatory effect of xenon was the largest. Microglia plays a major role in the pro-inflammatory cytotoxic response and participates in the immunosuppressive processes contributing to further tissue damage (29). We postulate a possible immunomodulatory mechanism of xenon reducing microglial activation and contributing to a decrease in neuronal cell damage.

Although not significant ($p = 0.168$), it is unclear why the Sham Xe group presented a higher summed-up score and thus more hippocampal damage, compared to the Sham $N_2$ group. It could be that Xenon has no effect on the background damage occurring in our Sham animals indicating that the neuroprotective effect of Xenon cannot be generalized.

We have previously demonstrated a reduction in mortality after argon post-conditioning in a SAH animal model (26). Here, we present for the first time the beneficial effect of xenon treatment after experimental SAH with a reduction of hippocampal neuronal cell loss and a decrease in cortical microglial cell activity. Until now, the neuroprotective effects of xenon have been accredited to the inhibition of the NMDA receptor (30–32). *In vitro* research has shown that NMDA receptor stimulation triggers microglia activation and the secretion of neurotoxic factors (33). This could very well explain the immunomodulatory mechanism observed in our xenon-treated animals. Xenon may be a potential clinical treatment for EBI under carefully defined conditions. By attenuation of the complex inflammatory mechanisms, some of the devastating secondary injuries may be prevented and outcome in SAH patients could be improved.

### Limitations

The primary weakness of our trial was the limited number of included animals. We estimated that a higher number of included animals could demonstrate a more pronounced therapeutic effect. Second, per ROI, only a single HPF was evaluated for calculation of neuronal cell loss (in H&E and NeuN Staining) and for the evaluation of microglial activation in Iba-1 staining. In the initial trial design, neuronal cell loss was manually counted in the entire hippocampus. As the trial continued, we observed a good congruence between these results and the estimated damage using only a single HPF. Additionally, to estimate neuronal cell loss, we only looked at the right hippocampus. This was done because the insertion of the ICP probe on the left side caused additional damage to the left hemisphere introducing potential bias. ROIs in the left hemisphere were included for the assessment of microglial activation since they were located at the base of the brain, farther away from the regions of iatrogenic damage.

Until now, we did not examine whether the xenon-mediated reduction in Iba-1-positive cells coincided with a reduction in cortical damage. Because of its higher cell count, estimating cortical damage is more time consuming but could be the focus of future research projects. Additionally, it is worth mentioning that Iba-1 is not a specific marker for microglia and unspecific binding of the antibody could have compromised our results.

## CONCLUSION

This is the first time that a neuroprotective effect of xenon has been shown. The effect is potentially mediated by an inhibitory effect on microglial activation. Further animal research should focus on long-term clinical outcome post Xenon ventilation in a SAH model.

## ETHICS STATEMENT

The study protocol was approved by the government agency for animal use and protection (Protocol number: TVA 10416G1 approved by "Landesamt für Natur, Umwelt und Verbraucherschutz NRW," Recklinghausen, Germany), all experiments were conducted in accordance with the Guide for Care and Use of Laboratory Animals (National Research Council, and the Committee for the Update of the Guide for the Care and Use of laboratory Animals; 8th edition 2011).

## AUTHOR CONTRIBUTIONS

Animal experiments: AH. Data analysis: MV and AH. Manuscript drafting: MV. Manuscripts revision and editing: MV, MC, RR, HC, BK, and AH. Final approval of the manuscript: MV, MC, RR, HC, BK, and AH.

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MINI REVIEW
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# The Role of Thromboinflammation in Delayed Cerebral Ischemia after Subarachnoid Hemorrhage

*Devin W. McBride[1]*, Spiros L. Blackburn[1], Peeyush Kumar T[1], Kanako Matsumura[1] and John H. Zhang[2,3]*

*[1] The Vivian L. Smith Department of Neurosurgery, McGovern Medical School, The University of Texas Health Science Center at Houston, Houston, TX, United States, [2] Department of Physiology and Pharmacology, Loma Linda School of Medicine, Loma Linda University, Loma Linda, CA, United States, [3] Department of Neurosurgery, Loma Linda School of Medicine, Loma Linda University, Loma Linda, CA, United States*

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***Correspondence:**
*Devin W. McBride*
*devin.w.mcbride@uth.tmc.edu*

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Delayed cerebral ischemia (DCI) is a major determinant of patient outcome following aneurysmal subarachnoid hemorrhage. Although the exact mechanisms leading to DCI are not fully known, inflammation, cerebral vasospasm, and microthrombi may all function together to mediate the onset of DCI. Indeed, inflammation is tightly linked with activation of coagulation and microthrombi formation. Thromboinflammation is the intersection at which inflammation and thrombosis regulate one another in a feedforward manner, potentiating the formation of thrombi and pro-inflammatory signaling. In this review, we will explore the role(s) of inflammation and microthrombi in subarachnoid hemorrhage (SAH) pathophysiology and DCI, and discuss the potential of targeting thromboinflammation to prevent DCI after SAH.

**Keywords: thromboinflammation, delayed cerebral ischemia, subarachnoid hemorrhage, thrombosis, inflammation, cerebral vasospasm**

## INTRODUCTION

Aneurysmal subarachnoid hemorrhage (aSAH) affects 9 per 100,000 individuals per year in the United States (1) and has high rates of morbidity and mortality (2). Since the mean affected age is 45–55 years old (3), the socioeconomic burden is great, and despite advances in aneurysmal securement technique and management of aSAH in the last 20 years, many of those who survive the initial insult develop significant neurological and cognitive disabilities (1, 4–6).

A major contributor to poor outcomes after aSAH is delayed cerebral ischemia (DCI) (1, 5, 6). DCI affects 20–30% of survivors and occurs between 4 and 10 days after subarachnoid hemorrhage (SAH), leading to cognitive decline (7–9), ultimately causing severe disability and worse quality of life or death (10–12). DCI is the clinical syndrome used to describe delayed development of neurological impairment (9). Historically, DCI was thought to be caused by cerebral vasospasm. However, multiple studies indicate that DCI is multifactorial, with vasospasm being just a contributing factor (13–17) along with cortical spreading depression, disrupted/altered cerebral autoregulation, microthrombosis, and inflammation (6, 18). Due to the multi-faceted nature of DCI involving both inflammation and microthrombosis, in this review, we will provide evidence that thromboinflammation is an unexplored therapeutic target for preventing DCI in aSAH patients.

## SAH PATHOPHYSIOLOGY

Following the initial bleed and subsequent treatment of the ruptured aneurysm, the accumulated blood within the subarachnoid space triggers several response mechanisms, as well as pathological events. In response to extravascular blood, endogenous mechanisms of repair are initiated, including removal of the red blood cells (RBCs) (19). To this end, specific cell adhesion molecules are rapidly expressed on the luminal endothelial cell surface (19), which allows circulating macrophages and neutrophils to enter into the subarachnoid space. Extravasated blood is phagocytosed by infiltrating macrophages (20), and as a result, inflammatory cytokines are released by the infiltrated leukocytes and activated resident microglia, stimulating the immune cascade. Indeed, inflammation has been shown to be a major player in early brain injury (21, 22), and may be a factor leading to DCI (23, 24), contributing to long-term deficits (25–27).

A second pathological factor triggered after SAH is hypercoagulability (28). Although the coagulation cascade is immediately activated following aneurysm rupture to stop bleeding, the hypercoagulable state does not end when the bleeding ceases; a sub-set of aSAH patients will continue to have hypercoagulability for several days post-SAH which has been shown to correlate with the development of DCI (28) and result in poorer outcome (28, 29).

## Mechanism of Thromboinflammation after SAH

While there are distinct mechanisms by which both inflammation and thrombosis can lead to deleterious events after SAH, both of these pathological events have cross talk by which they potentiate each other (30). The overlap between inflammation and thrombosis, called thromboinflammation, has been reported to play roles in a number of brain diseases/damage, including ischemia (31–34). Since ischemia also plays a role in patient outcome as a downstream deleterious event from SAH, thromboinflammation may be an overlooked pathophysiology after SAH.

Following SAH, activated macrophages release pro-inflammatory cytokines/chemokines for the recruitment of circulating macrophages to aid in RBC clearance (19). Phagocytosis of RBCs and subsequent death of the infiltrated leukocytes (occurring 2–4 days post-ictus) in the cerebrospinal fluid leads to release of a plethora of toxic molecules (35) including endothelins, oxygen free radicals, and hemoglobin/heme (and byproducts), thereby potentiating pro-inflammatory pathways and endothelial cell-mediated thrombosis. The latter event induces a positive feed-forward loop, potentiating thromboinflammation by continuing to recruit systemic immune cells for debris clean-up, causing cell adhesion molecules to continuedly activate platelets, leading to microthrombi formation (36) (**Figure 1**).

Production/release of reactive oxygen species and nitric oxide adds to endothelial damage (37), stimulating endothelial expression of adhesion molecules (e.g., ICAM-1, P-selectin, VCAM, collagen, fibrinogen, etc.) (37, 38), and release of tissue factor and vWF, thereby activating platelets. This process is controlled by ADAMTS13 (a disintegrin-like metalloprotease with thrombospondin type 1 repeats-13), which acts to control clot formation by cleaving hyperactive ultralarge vWF to prevent platelet adherence (39). Interestingly, aSAH patients who go on to develop DCI are more hypercoagulable than aSAH patients who will not develop DCI (40, 41). Auxiliary to greater/sustained microthrombi formation, continued activation of coagulation by endothelial cells and platelets further promote inflammation *via* activation of circulating leukocytes through protease activated receptor-1 and toll-like receptor 4 signaling (36).

Finally, emerging evidence further displays the delicate balance that inflammation plays in modulating coagulation (36). In the non-diseased state, systemic leukocytes aid in preventing coagulation by expressing several anticoagulant factors (42–44). Yet, when pro-inflammatory signaling is initiated, leukocytes change phenotypes, thereby aiding in coagulation through the release of procoagulant factors (45, 46) and reduced expression and degradation of anti-coagulation factors (47–49). While leukocytes play a critical role in inducing and propagating inflammation, leukocytes also promote a hypercoagulable state after SAH (36) and may play a crucial role in DCI *via* thromboinflammation.

### Clinical Evidence

To date, the exact role thromboinflammation plays in patients suffering from aSAH is not known. Yet, the following clinical studies suggest that thromboinflammation may be a key contributor to DCI after aSAH, and thus should be considered as a therapeutic target.

First, microthrombi have been reported as an associative cause of DCI and worse neurological outcome (50, 51). In human autopsy studies, microthrombi, reported to be throughout the brain, are associated with regions of infarct (50, 51), and also develop with a timing similar to DCI (6). Following aSAH, platelet activation is over-stimulated, resulting in microthrombi within small arterioles (51), peaking within 2 days and then again from 1 to 2 weeks post-ictus (50). Platelet-derived thromboxane B2 is higher and platelet count is lower in DCI patients compared to non-DCI patients (52, 53), suggesting platelet aggregation as a potential cause of microthrombi. In addition, aSAH patients are hypercoagulable with changes in platelet-activating factor (54), vWF (40, 54), and tissue plasminogen activator (55, 56) correlating with the incidence of DCI (54, 55) and poor patient outcome (28). Specific polymorphisms of plasminogen activator inhibitor (PAI) have higher PAI activity (57) leading to a higher incidence of DCI (41). Finally, in addition to an over active coagulation cascade, fibrinolysis after aSAH is impaired (40, 56). ADAMTS13, proposed as a critical link between inflammation and thrombosis (39), has been observed to have low activity after aSAH, and is associated with an increased risk of ischemic stroke (58) and may potentiate microthrombi formation. The inherent roles of leukocytes and platelets in inflammation and thrombosis, as well as the involvement of ADAMTS13, argue for thromboinflammation being considered as a therapeutic target after SAH.

Not only is the coagulation cascade not functioning properly, so too is inflammation continually hyperactive. Several pro-inflammatory cytokines have been linked with worse clinical outcome after aSAH (25–27, 59, 60). Increases in tumor necrosis factor α, interleukin-1α, interleukin-1β, interleukin-6, and

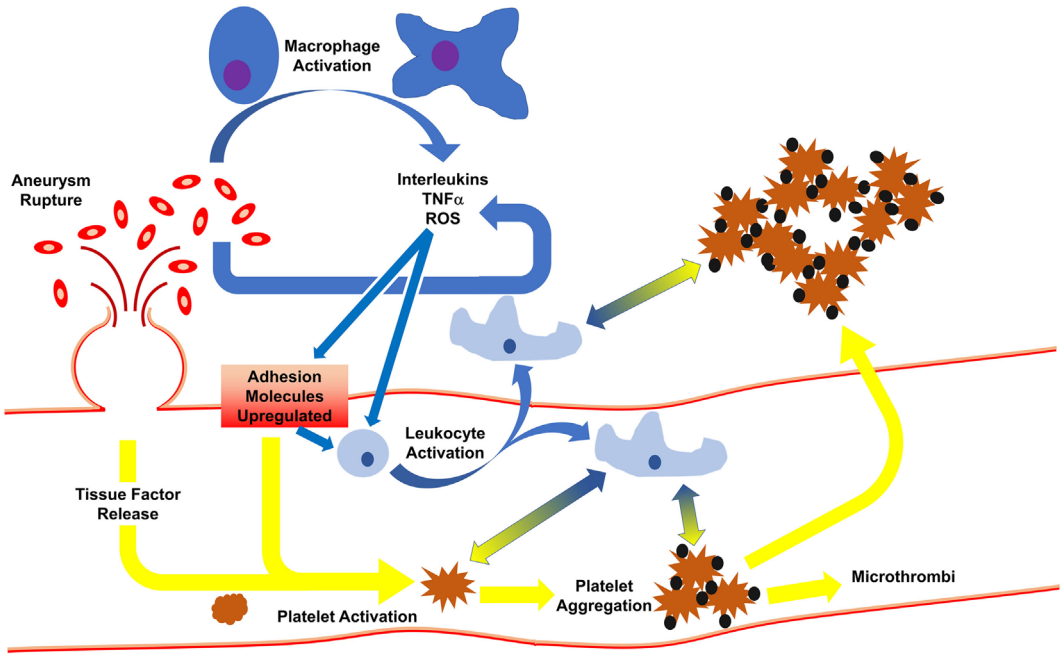


**FIGURE 1** | Initiation of inflammation and thrombosis after subarachnoid hemorrhage. Following rupture of an aneurysm, red blood cells spill into the subarachnoid space, activating macrophages which release cytokines. In response, endothelial cells express adhesion molecules to activate circulating leukocytes and platelets. This results in leukocyte infiltration, phagocytosis of red blood cells, and release of more cytokines. Activated platelets develop into thrombi which may travel downstream to occlude distal microvessels or may cross into the brain parenchyma. Platelet activation and aggregation signal for leukocyte activation, and vice versa, such that even when the aneurysm is treated, thromboinflammation continues *via* feedforward mechanisms.

interleukin-8 have all been found in the cerebrospinal fluid and blood (59, 61–68), with several factors also being associated with poor outcome and DCI (59, 61). Additionally, elevated adhesion molecules in the blood have also been observed after aSAH in patients, remaining elevated for 6–8 days, and are associated with DCI (69–71). Blood P-selectin is elevated in patients with DCI, suggesting that inflammation and platelet activation adhesion are associated with DCI (72, 73). Indeed, adhesion molecules are well documented to be at the crux of thromboinflammation (39, 74–76). These findings, which suggest thromboinflammation is active, are also supported by increased leukocyte infiltration into the brain following SAH (77–80) and also correlate with ischemia after SAH (81). Thus, the so-called "leukocyte–endothelial cell interaction" (78) may play a role in SAH pathophysiology (i.e., thromboinflammation).

A recent study on 106 aSAH patients showed that platelet activation and inflammation (*via* C-reactive protein measurement) occurred simultaneously and were associated with worse early brain injury and 3-month outcome (82). Similar to others (40, 54), Frontera et al. found that platelet activation and C-reactive protein levels were associated with DCI, but even more interesting, the authors reported that higher platelet activation and C-reactive protein levels at 72 h post-SAH correlate with DCI (82). The findings by Frontera et al. further support the potential role of thromboinflammation in DCI after aSAH, suggesting thromboinflammation may be an unexplored therapeutic target for improving aSAH outcome.

## Evidence from Experimental Models

Thromboinflammation has also not been specifically studied in experimental models of SAH, nor has it been examined for correlation with microthrombi, ischemia, or DCI in animals. While DCI is unmeasurable in animals, several pathological measures are used as correlates for DCI (e.g., vasospasm, microthrombi formation, ischemia/infarct/cell death).

Several groups have reported the existence of microthrombi within the vasculature both locally, as well as distally from the SAH insult in experimental SAH models (83–86). In rodent models of SAH using autologous blood infusion, microthrombi and occluded blood vessels have been reported to occur throughout the brain at 2 days post-SAH (84) and can be observed for up to 7 days post-ictus (86). While microthrombi have been observed in puncture models of SAH (87, 88), it is the blood infusion models which offer the most insight into the potential role of thromboinflammation in clot formation after experimental SAH. Indeed, thrombi may be thrown downstream from the vessel rupture in endovascular perforation models as the mechanism by which microthrombi occur in the vasculature; however, no such vessel rupture is present in the autologous blood infusion models. In the blood injection SAH models, the likely cause of microthrombi formation and deposition within the cerebral vasculature distal from the SAH insult is inflammation.

Furthermore, following experimental SAH, endothelial damage resulting from leukocyte infiltration may lead to the downward spiral that is thromboinflammation. Indeed,

post-SAH inflammation is devastating (19) and includes cytokine/chemokine upregulation (89, 90), increased expression of adhesion molecules (91, 92), and leukocyte infiltration (93–95). In fact, either depleting neutrophils or reducing neutrophil activity decreases microvascular injury following SAH in rats (93).

Similar to clinical findings, P-selectin has been observed to increase after experimental SAH, leading to activation of platelets and thrombi formation (84). Interestingly, platelet aggregates have been observed to extravasate into the brain parenchyma in the rodent SAH autologous blood injection model which likely propagates pro-inflammatory signaling (22), further showing that there is cross talk between inflammation and thrombosis after SAH.

Evidence from experimental models of SAH, similar to clinical findings, suggest that significant overlap between inflammation and thrombosis occurs post-SAH and may be involved in secondary injury and neurological deterioration. Future studies are warranted to investigate the exact role(s) thromboinflammation plays in SAH and its potential as a therapeutic target.

# THROMBOINFLAMMATION AS A THERAPEUTIC TARGET TO PREVENT DCI

The detrimental roles thromboinflammation play in brain pathologies has led to thromboinflammation to be suggested as a therapeutic target (32, 96), but not for SAH. Based on the review presented above, thromboinflammation should also be considered a therapeutic target for patients of SAH (54). Potential targets of thromboinflammation may include expression of adhesion molecules, release of factors activating leukocytes and platelets simultaneously (e.g., selectins), disrupting platelet-leukocyte formations, and targeting ADAMTS13. To date, no clinical trials have studied these factors as specific therapeutic targets. Below we review some of the clinical trials which have targeted either coagulation or inflammation, and show that targeting these physiological events separately does not seem to hold the answer for SAH treatment.

## Previous Trials

### Targeting Coagulation and Clot Clearance

Several early clinical trials investigated therapeutics directed at targeting coagulation following SAH, many of which used antiplatelet therapies. However, meta-analysis of antiplatelet therapy indicates that antiplatelet therapy has a trend for improving outcome in aSAH patients, with tendencies to decrease secondary ischemia (except for acetylsalicylic acid which shows no benefit) (97). Some of the antiplatelet therapies thus far investigated are acetylsalicylic acid, ticlopidine (adenosine diphosphate receptor inhibitor), dipyridamole (phosphodiesterase inhibitor), OKY-046/Calaclot (thromboxane synthetase inhibitor), a thromboxane A2 antagonist, and E5880 (inhibitor of platelet-activating factor receptor) (97). Of these agents, Calaclot and E5880 seemed to improve functional outcome, but additional studies need to be undertaken to determine the therapeutic benefit associated with antiplatelet therapies.

Studies have also examined the benefit of targeting clot clearance. In this regard, recombinant tissue plasminogen activator (administered intraventricularly) has been investigated in several clinical trials which have shown that although recombinant tissue plasminogen activator can improve clot clearance, it fails to attenuate cerebral vasospasm or DCI (98), reduce mortality (99), or improve functional outcome (100). These findings indicate that clot clearance alone may not be enough for aSAH.

### Targeting Inflammation

Trials targeting inflammation have had varied success. Erythropoietin-β, despite having mechanisms of action against vasospasm and inflammation (101), had no effect on cerebral vasospasm, but did reduce DCI and improve functional outcome (102). This was a small study and is at high risk for bias (103), so additional trials are needed. Another anti-inflammatory agent, methylprednisolone, was found to improve functional outcome at 1 year despite having no effect on cerebral vasospasm or hypodensity on CT scans (104). It should be noted that methylprednisolone is an anti-inflammatory and thus may have no effect on vasospasm, although the lack of change in CT hypodensity is puzzling. Regardless, these trials suggest that inflammation indeed plays a major role in SAH outcome. Since there is considerable feedforward/back mechanisms between thrombosis and inflammation, anti-inflammatory agents may indirectly reduce thromboinflammation. While previous trials have shown promise, additional studies are needed to determine any benefit to patient outcome for anti-inflammatory agents as well as the relationship to thromboinflammation.

## Current Trials

The lack of promising data for therapeutics targeting either cerebral vasospasm (100, 103) or clot clearance suggest that SAH prognosis may lie within inflammation, platelet activation, or at the intersection of the two (i.e., thromboinflammation). To date, the only FDA approved treatment after aSAH is nimodipine. While the initial mechanism of action was purported to be *via* anti-vasospasm (105, 106), its effects seem to be more than just preventing vasospasm. Indeed, nimodipine can improve patient outcome irrespective of vasospasm attenuation (107). Although the exact mechanism of nimodipine is unknown, it may reduce microthrombosis, be neuroprotective, and inhibit cortical spreading ischemia (14, 108). Furthermore, nimodipine has been implicated as being anti-inflammatory (109), and it is important to note that calcium channel blockers may reduce leukocyte infiltration (110), and therefore reduce thromboinflammation. Mechanistic studies for the role(s) of nimodipine are needed to clarify the mechanisms by which nimodipine improves patient outcome after SAH. These studies may also shed light onto the therapeutic targets for thromboinflammation.

Fasudil is currently used in Asia instead of nimodipine, because the latter is not commercially available. Fasudil is a rho-kinase inhibitor which is reported to reduce hemodynamic dysfunction (i.e., vasoconstriction, endothelial injury) and inflammation through downstream signaling pathways *via* inhibition of rho-kinase (111). Initially, fasudil was used to target cerebral vasospasm, but fasudil was found to also reduce cerebral infarction,

and improve functional outcome after aSAH (112). Thus, fasudil also seems to provide therapeutic benefit for aSAH patients, but still requires validation in large randomized controlled clinical trials (113). Being a rho-kinase inhibitor, fasudil may prevent a number of downstream signaling pathways involved in SAH pathophysiology [smooth muscle cell contraction and endothelial dysfunction (95, 114), inflammation, and leukocyte activation (115)], arguing that drugs with pleiotropic effects may be an answer. Similar to nimodipine, the exact mechanisms by which fasudil improves outcome following SAH remain a mystery, and translational studies for SAH will benefit for additional studies on the mechanism(s) of fasudil.

The recent study by Wessell et al. found that infusion of low-dose heparin administered with nimodipine is associated with increased odds of patient discharge (116), and, in a preliminary trial, heparin reduced cerebral vasospasm and infarct (117). These studies support the notion that combining therapies which reduce distinct injury mechanisms can provide increased effectiveness. Furthermore, these studies indicate that there may be therapeutic potential in anti-coagulation and anti-inflammation (103). Currently, heparin is currently being explored in the ASTROH trial (NCT02501434) which will examine the efficacy of heparin for reducing vasospasm and delayed neurological deficits in SAH patients.

Specific information regarding the role of thromboinflammation and its potential as a therapeutic target may be elucidated through the SoSTART trial (NCT03153150) and the etanercept (TNFα receptor antagonist) trial (NCT01865630). The SoSTART trial is set to investigate seven anti-coagulants for therapeutic benefit in patients of cerebral hemorrhage, including aSAH. These proposed drugs are known to be reduce coagulability and prevent inflammation (118, 119), which may reduce the overall thromboinflammation.

### Challenges in Targeting Thromboinflammation

Several challenges exist when considering thromboinflammation as a therapeutic target for preventing DCI. First and foremost, special care needs to be taken when identifying potential therapies for thromboinflammation since the ideal candidate will only target newly formed microthrombi and not have any significant effect on the clot formed at the site of aneurysm rupture. Once the aneurysm has been secured with clip or coil, the aneurysm is of less concern, but potential complications such as post-operative bleeding or bleeding along the ventriculostomy remain a risk.

In most pathologies, including SAH, both pro- and anti-inflammatory pathways are activated to promote debris/toxin clearance and repair of injured tissue, respectively. Since pro-inflammatory cytokines and downstream signaling is linked with thromboinflammation (34), it is critical to pursue agents which mitigate pro-inflammatory cytokines. So far research has largely focused on this, however, anti-inflammatory signaling aids in repair and healing, thus this arm of inflammation should remain active (or upregulated) after reducing thromboinflammation.

Finally, although experimental studies are required to understand pathophysiology, uncover signaling mechanisms, and investigate novel drugs and potential therapeutic targets, animals (including non-human primates) have differences when it comes to coagulation (120, 121), as well as inflammation (122, 123). Therefore, it follows that animals may have distinct differences when it comes to thromboinflammation and its treatment. Future studies should examine these differences to aid in therapeutic development against thromboinflammation.

## CONCLUSION

Thromboinflammation, the cross talk between the thrombotic and inflammatory pathways, likely plays a critical role in the development of DCI after SAH, thereby having significant impact on overall patient outcome. The current understanding of thromboinflammation and its role in SAH pathophysiology is only beginning to emerge, but evidence from experimental and clinical studies suggest that preventing thromboinflammation has the potential for benefiting patients of aSAH.

## AUTHOR CONTRIBUTIONS

All authors contributed to the conception and drafting of this article.

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HYPOTHESIS AND THEORY
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# Hypothesis on the Role of Cryptochromes in Inflammation and Subarachnoid Hemorrhage Outcome

*Adriano Barreto Nogueira[1,2]*, Ariel Barreto Nogueira[3], José Carlos Esteves Veiga[2] and Manoel Jacobsen Teixeira[1,4]*

*[1] Division of Neurosurgery Clinic, Hospital das Clinicas, Faculty of Medicine, University of Sao Paulo, Sao Paulo, Brazil, [2] Discipline of Neurosurgery, Santa Casa Faculty of Medical Sciences, Department of Surgery, Sao Paulo, Brazil, [3] Faculty of Medicine, Department of Radiology, Hospital das Clinicas, University of Sao Paulo, Sao Paulo, Brazil, [4] Faculty of Medicine, Department of Neurology, University of Sao Paulo, Sao Paulo, Brazil*

We have recently found that the temperature variability (TV) in the day–night cycle may predict the mean intracranial pressure in the following 24 h ($ICP_{24}$) in subarachnoid hemorrhage (SAH) patients under multimodality monitoring, sedation, and hypothermia (<35°C). Specifically, we found that $ICP_{24}$ = 6 (4 − TV) mmHg. TV is the ratio between the coefficient of variation of temperature during the nocturnal and the preceding diurnal periods. This result suggests that the circadian clock reflects brain plasticity mechanisms and its malfunctioning leads to deterioration of the neurologic status. The sleep–wake cycle is absent in these patients and their circadian clock can function properly only by environment light-independent mechanisms. One mechanism involves the circadian clock proteins named cryptochromes (CRYs). CRYs are highly preserved and widespread in the evolutionary tree, are expressed in different cell types in humans [type II CRYs, in two forms: human cryptochrome 1 and 2 (hCRY1 and hCRY2)], and in certain species, respond to blue light and play role in magnetoreception. Interestingly, SAH outcome seems to correlate with inflammation, and CRYs decrease inflammatory activity. Our hypothesis derived from these observations is that CRYs modulate the circadian oscillation of temperature even during therapeutic hypothermia and improve outcome in SAH through decrease in inflammation. A strategy to test this hypothesis is to measure periodically during the acute phase of high-grade SAH the level of CRYs in cerebrospinal fluid (CSF) and circulating white blood cells, and to correlate these levels with outcome, TV, $ICP_{24}$, and pro- and anti-inflammatory markers in CSF and blood. If this hypothesis is true, the development of therapies targeting inflammation in SAH could take advantage of cryptochrome properties. It has been shown that blue light phototherapy increases the expression of CRYs in blood mononuclear cells in jaundiced neonates. Likewise, visual stimulus with flashing light improves Alzheimer's disease features in experimental model and there is a prominent expression of CRYs in the retina. Remarkably, recent evidence showed that hCRY2 responds to electromagnetic fields, which could be one elusive mechanism of action of transcranial magnetic stimulation and a reason for its use in SAH.

**Keywords: cryptochromes, subarachnoid hemorrhage, inflammation, neurogenesis, circadian rhythm, intracranial pressure prediction, therapeutic hypothermia, targeted temperature management**

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****Correspondence:***
*Adriano Barreto Nogueira*
*adriano.nogueira@hc.fm.usp.br*

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## INTRODUCTION

Our previous work indicates that circadian rhythms are a primary factor to predict brain injury (1–6). First, we showed that the anterior hypothalamus displays a prominent expression of neurogenesis-related markers in adult humans (5, 6). The functions of the anterior hypothalamus include circadian rhythm control and thermoregulation. Because of these features, we suspected that the monitoring of circadian oscillation of temperature could measure putative brain regeneration and plasticity mechanisms and, therefore, anticipate the intensity of brain injury. Following this rationale, we discovered that the ratio between the mean temperature during the sleep and the preceding wake period correlates with the likelihood of seizure occurrence in the following 24 h in epileptic patients (1, 3). We have recently showed that this concept works even with high-grade subarachnoid hemorrhage (SAH) patients under sedation and therapeutic hypothermia induced by intravascular catheter (2). In this case, the ratio between the coefficient of variation of temperature during the nocturnal and the preceding diurnal periods correlates with the mean intracranial pressure in the following 24 h ($ICP_{24}$) (2). Collectively, these findings open the possibility to reduce brain injury through circadian rhythm modulation.

Circadian rhythms may be modulated by interfering on the level of the clock proteins cryptochromes (CRYs). CRYs are evolutionarily old flavoproteins expressed in two types, in species ranging from plants to humans (7). Mammals display ubiquitous expression of type II CRYs (8, 9). In humans, there are two forms of type II CRYs, named human CRY1 (hCRY1) and CRY2 (hCRY2) (8, 9). CRYs are highly expressed in the suprachiasmatic nucleus (10). The suprachiasmatic nucleus is the hypothalamus structure that orchestrates the circadian rhythms by the central nervous system. CRYs also interfere in the other mechanism of circadian rhythm control (peripheral mechanism), which includes immune system cells (7). Importantly, hCRY levels can be potentially modulated by blue light (11) and electromagnetic field (8), which are currently used in clinical practice.

## HYPOTHESIS

We hypothesize that the ratio between the level of expression of hCRYs during the nocturnal and the preceding diurnal periods ($CRY_{n/d}$) correlates with inflammation and predicts neurologic signs in the following day–night cycle and long-term outcome in SAH.

## RATIONALE OF THE HYPOTHESIS

### Adult Human Neurogenesis

The discovery of adult mammalian neurogenesis opened a novel perspective on brain plasticity and neuroregeneration therapies (4–6). The current prevailing concept that the adult mammalian brain harbors two primary neurogenic niches in the subgranular zone of the dentate gyrus and in the subventricular zone replaced the long-lasting dogma of no-new neurons after birth (4–6). Adult human neurogenesis is controversial, but we have previously shown that the adult human brain harbors what we referred to as potential neurogenic system (4–6). The potential neurogenic system was revealed by the detection of expression of neural stem cell markers in the circumventricular organs (4–6). The circumventricular organs are the brain structures located principally in the hypothalamus that display no blood–brain barrier. Moreover, brain structures adjacent to the circumventricular organs, which form part of the hypothalamic and limbic system circuits, express the immature or newly formed neuron marker doublecortin (DCX) (5, 6). We interpreted these results as a potential constitutive mechanism of neurogenesis that could participate in the maintenance of certain brain circuits and in their regeneration after injury. Next, we pursued a method to measure the effects of this potential plasticity mechanism in clinical practice.

### Brain Injury Prediction through Circadian Rhythm Monitoring

#### Presence of Sleep–Wake Cycle

Because the hypothalamus displays robust expression of neurogenesis-related markers and modulates circadian rhythms and temperature, we suspected that these functions parallel endogenous brain plasticity. Indeed, alterations in the hypothalamus-related functions anticipate worsening in neurologic status, maybe partially due to alterations in endogenous neurogenesis mechanisms. In practice, we found that the ratio between the mean skin temperature during sleep and the preceding wake period ($T_{s/w}$) correlates with the likelihood of seizure occurrence in the following 24 h in epileptic patients (1, 3). This method is non-invasive and involves the use of wristband with sensors of vital signs (1, 3). Importantly, skin temperature has been shown to be a reliable circadian rhythm marker (1, 3).

#### Absence of Sleep–Wake Cycle

A stronger indication that the circadian rhythm is a primary factor to predict brain injury is being unveiled by our ongoing research showing that the pattern of oscillation of core body temperature in the day–night cycle in high-grade SAH patients correlates with further intracranial pressure (2). The patients analyzed in this study underwent sedation and targeted temperature management (33–33.9°C) using intravascular catheter. Circadian rhythm correlated with further neurologic signs even in this situation of decreased level of consciousness, lack of sleep–wake cycle, and strict temperature control. Specifically, the ratio between the coefficient of variation of the nocturnal (starting at 18:00) and the preceding diurnal periods (starting at 6:00) [temperature variability (TV)] correlated with $ICP_{24}$ ($p < 0.001$). The formula derived from regression analysis is $ICP_{24} = 6\,(4 - TV)$ mmHg (2).

The findings of our line of research indicate the existence of a light-independent mechanism of circadian rhythm that participates in brain plasticity (9, 12). The discovery of a factor involved in this mechanism could lead to the development of a circadian rhythm-guided therapy to prevent brain injury.

### Cryptochrome As Potential Prediction Factor in SAH

Cryptochromes display circadian expression in the suprachiasmatic nucleus (10) and in peripheral tissues (7) such as white

blood cells (11) through light-independent (7, 9) and light-dependent mechanisms (8, 10, 12).

The expression of hCRYs decreases during the day and increases during the night (13). Insufficient increase in hCRYs during the night correlates with higher levels of inflammation in the following morning, as has already been demonstrated in rheumatoid arthritis patients (13). In SAH patients, inflammation seems to lead to poor outcome (4).

The circadian pattern of temperature oscillation correlates with further neurologic signs (i.e., seizure in epileptic patients and intracranial hypertension in high-grade SAH) (1–3), and an elusive mechanism of this finding is that a normal circadian rhythm reflects normal hypothalamic neurogenesis (**Figure 1**) (1–6). Intriguingly, another potential marker of circadian rhythm, which could theoretically form a loop involving hCRYs and neurogenesis is blue light emission by the body. Endogenous blue light emission has been shown from human body (14). This emission displays circadian pattern and correlates with metabolism and reactive oxygen species (ROS) formation (14). Blue light stimulates CRY expression (11), and both—CRY (15) and ROS (16)—stimulate neurogenesis. Conversely, neurogenesis could close the loop by decreasing ROS formation, blue light emission, and CRY stimulation.

These observations raise the possibility that the circadian pattern of CRY expression correlates with circadian patterns of markers of inflammation and neurogenesis, neurologic signs in the following day–night cycle, and outcome in high-grade SAH.

## HYPOTHESIS TESTING

The primary analysis to test our hypothesis is the correlation of the ratio between the level of hCRYs in the cerebrospinal fluid (CSF) during the nocturnal and the preceding diurnal periods ($CRY_{n/d}$) of acute phase of high-grade SAH patients with outcome after 1 year. This level can be determined by immune-enzymatic assay (ELISA). In addition, bearing in mind the relationship between CRYs and inflammation, the ratio can be calculated also regarding the expression of hCRYs in white blood cells ($WBCC_{n/d}$) (and their different types, e.g., mononuclear cells).

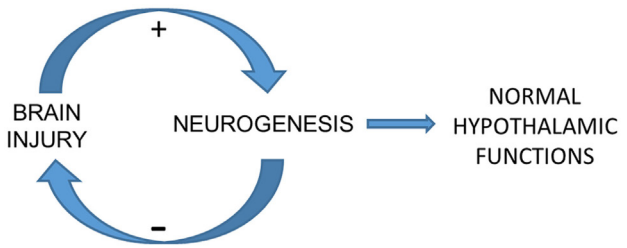


**FIGURE 1** | Elusive mechanism of the correlation between cryptochrome expression, temperature variability (TV), and further intracranial pressure (ICP). The potential neurogenic system that we described recently may be a background mechanism of brain plasticity whose core is located in the hypothalamus. This system may be modulated by the redox state of brain cells, which is remarkably altered in brain injury. An effective injury-induced neurogenesis would restore hypothalamus-related functions such as circadian rhythms and next autonomic functions related to cerebral autoregulation, contributing to ICP control. TV seems to be a marker of circadian rhythm even in comatose and sedated patients undergoing hypothermia. This condition implies the role of a light-independent mechanism of circadian rhythm control, which may be played by hCRY expression.

Due to potential therapeutic implications of the hypothesis (as detailed below), a crucial analysis is the correlation between $CRY_{n/d}$ and TV and $ICP_{24}$. A caveat of this analysis is that TV has been shown to correlate with $ICP_{24}$ only under strict control of temperature, with daily mean <35°C (2).

The complete study to test the hypothesis proposed here includes our published protocol to assess multimodality monitoring, inflammation, and neuroregeneration markers in CSF and blood of high-grade SAH patients aiming at revealing a prognostic biomarker (4). Because of our previous work, we deem worthwhile including in our original protocol principally the analysis (from blood and CSF samples of SAH patients) of markers related to circadian rhythms (melatonin), thermoregulation (RANK) (17), and hypothalamus- or hypophysis-produced hormones (growth hormone-releasing hormone, growth hormone, gonadotropin-releasing hormone, and oxytocin) (**Table 1**). Accordingly, the pineal gland and neurohypophysis are circumventricular organs, and the hormones mentioned above stimulate neurogenesis (18, 19).

Also bearing in mind its intriguing presence in the circumventricular organs [pineal gland (20)/habenular zone (21), choroid plexus (22), hypothalamus parenchyma (22)], it would be interesting to quantify through cell sorting mast cells in the CSF of the patients enrolled in this type of protocol. The potential neurogenic system arises from zones without blood–brain barrier, whose permeability is dynamic across the sleep–wake cycle (23). An indication that mast cells may contribute to dynamic features of blood–brain barrier is that they contain granules of histamine, which increases locally the permeability of blood–brain barrier (21, 22). Histamine also decreases inflammation *via* activation of H2 receptor and is involved in neuroprotection associated with preconditioning (24). Furthermore, histamine increases neural stem cell proliferation and favors cell fate toward neuronal differentiation *via* activation of neural stem cell H2 and H1 receptors, respectively (24). The presence of mast cells in circumventricular organs, action of histamine to increase blood–brain barrier permeability, decrease inflammation, protect neurons from secondary injury, and stimulate neurogenesis suggest that mast cells may play a role in endogenous mechanisms of neuroprotection and neuroregeneration.

## EXPECTED RESULTS

In decreasing order of importance, the expected results for the study protocol summarized above are:

- $CRY_{n/d}$ correlates with SAH outcome after 1 year;
- $CRY_{n/d}$ correlates with TV and $ICP_{24}$;
- $CRY_{n/d}$ correlates with CSF and/or blood biomarker(s) of inflammation or neuroregeneration.

Some remarks should be mentioned regarding hypothesis testing and expected results (**Table 1**).

**TABLE 1** | Parameters for correlation analysis with CRYn/d.

| Parameter | Method | Function | Timing after night CRY assessment (18:00) |
|---|---|---|---|
| **Molecular and cellular markers in blood or CSF** | | | |
| **$WBCC_{n/d}$** | **qRT-PCR** | **Systemic circadian CRY oscillation** | **−12 and 0 h** |
| $WBCC_{n/d}$ (mononuclear) | qRT-PCR | Systemic circadian CRY oscillation | −12 and 0 h |
| Blood and CSF, interleukin-6, TNF-α, and CRP | ELISA | Inflammation | 12 and 24 h |
| Blood and CSF IL-10 and $T_h$ | ELISA and FACS | Anti-inflammatory | 12 and 24 h |
| CSF MCP-1 and SDF-1 | ELISA | Inflammation/neurogenesis | 12 and 24 h |
| Blood and CSF histamine | ELISA | Neuroprotection/neurogenesis | 12 and 24 h |
| CSF microglia and mast cells | FACS (CD-68 and CD-117/c-kit) | Inflammation/neurogenesis | 12 and 24 h |
| CSF CD133+ cells | FACS | Neurogenesis | 12 and 24 h |
| CSF ATP and ADP | ELISA | Redox state/purinergic pathway | −12 and 0 h |
| CSF cytochrome c and phosphoethanolamine | ELISA | Redox state/mitochondrial respiration | −12 and 0 h |
| CSF hydrogen sulfide | ELISA | Neuroprotection/neurogenesis | −12 and 0 h |
| CSF neuroglobin | ELISA | Oxygen metabolism | −12 and 0 h |
| Blood and CSF GH, GHRH, GnRH, oxytocin, vasopressin, and melatonin | ELISA | Circumventricular organ (median eminence, neurohypophysis, pineal gland) function | −12 and 0 h |
| CSF RANK | ELISA | Thermoregulation | −12 and 0 h |
| **ICU multimodality monitoring** | | | |
| **TV** | **Continuous core body T monitoring** | **$ICP_{24}$ prediction** | **−12 to 12 h** |
| **$ICP_{24}$** | **Continuous ICP monitoring** | **Intracranial hypertension** | **12 to 36 h** |
| Brain anatomy | CT scan | DCI | Clinical indication |
| $P_{ti}O_2$ | Parenchymal probe | Brain hypoxia | 12 to 36 h |
| Lactate/pyruvate | Microdialysis | Brain metabolism | 12 to 36 h (3 h intervals) |
| $MCA_v$, Lindegaard index, $CO_2$ reactivity | Transcranial Doppler | Vasospasm/cerebral autoregulation | 12 and 24 h |
| Alpha–delta ratio | Continuous EEG | DCI prediction | −12 to 12 h |
| **Outcome** | | | |
| **mRS** | **Clinical assessment** | **Functional** | Discharge, 1, 3, 6, and **12mo** |
| GOS | Clinical assessment | Functional | Discharge, 1, 3, 6, and 12mo |
| Barthel index | Clinical assessment | Functional | Discharge, 1, 3, 6, and 12mo |
| MMSE | Clinical assessment | Cognitive | Discharge, 1, 3, 6, and 12mo |
| MoCA | Clinical assessment | Cognitive | Discharge, 1, 3, 6, and 12mo |
| Sickness Impact Profile | Clinical assessment | Health-related QoL | Discharge, 1, 3, 6, and 12mo |
| Short-form 36 | Clinical assessment | Health-related QoL | Discharge, 1, 3, 6, and 12mo |

*CSF collection at 6:00 and 18:00 at minimum every 3 days for $CRY_{n/d}$ determination when external ventricular drain is placed, until 14 days after bleeding. Minimum, maximum, and mean $CRY_{n/d}$ for each patient should be compared with any of the remaining parameters to search for correlation. $WBCC_{n/d}$ and mononuclear WBC CRY levels can be used as an alternative parameter for circadian CRY level. Main parameters are in bold. The remaining parameters are options that can be selected according to the goal of the protocol or resources of the service.*

*ADP, adenosine diphosphate; ATP, adenosine triphosphate; CRP, C-reactive protein; CRY, cryptochrome; $CRY_{n/d}$, ratio between CRY level at night (18:00) and the preceding day (6:00) in CSF; CSF, cerebrospinal fluid; DCI, delayed cerebral ischemia; EEG, electroencephalogram; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescent-activated cell sorting; GH, growth hormone; GHRH, growth hormone-releasing hormone; GnRH, gonadotropin-releasing hormone; GOS, Glasgow Outcome Scale; ICP, intracranial pressure; $ICP_{24}$, mean ICP in the day following TV monitoring; ICU, intensive care unit; IL, interleukin; $MCA_v$, middle cerebral artery velocity; MMSE, mini-mental status exam; MCP, monocyte chemoattractant protein; MoCA, Montreal cognitive assessment; mRS, modified Rankin scale; $P_{ti}O_2$, brain tissue oxygen pressure; RANK, receptor-activator of nuclear factor κB; qRT-PCR, real-time polymerase chain reaction; QoL, quality of life; SDF, stromal cell-derived factor; T, temperature; $T_h$, T helper; TNF, tumor necrosis factor; TV, temperature variability; $WBCC_{n/d}$, ratio between CRY expression in white blood cells at night (18:00) and the preceding day (6:00).*

Cerebrospinal fluid collection for CRY determination will be carried out at 6:00 and 18:00, because they are the times of extreme core body temperature values. This procedure will be performed when an external ventricular drain is placed, at least every 3 days, and until 14 days after bleeding. For each patient, we will test three manners to calculate $CRY_{n/d}$: highest ratio, lowest ratio, and mean ratio of all daily $CRY_{n/d}$ values. CRY level will be calculated as hCRY1, hCRY2, and hCRY1 plus hCRY2 levels.

The role of inflammation in stroke and particularly in SAH pathophysiology is not totally established (25). For example, tumor necrosis factor alpha correlates with neuron damage or protection depending on the membrane receptor on which it acts (4). Interleukin-6 (IL-6) shows a similar dual effect depending on microenvironment conditions (4). Likewise, microglia participates in inflammation in the acute phase of stroke model, but in a later phase contributes to migration and differentiation of newly formed immature neurons (i.e., neuroblasts) (4).

The multifactorial nature of SAH pathophysiology hampers the determination of correlation between outcome and inflammation (25). SAH and brain injury trigger clinical features that lead to systemic inflammation (26), which on its turn may cause brain damage through seizures, for example (26). However, some studies showed that serum level of inflammation markers such as C-reactive protein (CRP) (27) and IL-6 (28, 29) correlates with SAH outcome.

Cerebrospinal fluid composition could in theory reflect a more straightforward level of brain inflammation, but factors such as amount of bleeding and use of external ventricular drain

may interfere on results. A stronger correlation in comparison with serum inflammation markers was demonstrated between CSF level of the inflammation-related marker high mobility group box-1 protein and SAH outcome after 3 months (30). This marker and CRP (27) also correlated with clinical (Hunt and Hess, World Federation of Neurological Societies, and Glasgow Coma scales) and radiological (Fisher) scales known to be prognostic factors in SAH.

The use of the ratio between CRY level during day and night is necessary to assess the role of circadian rhythm in SAH and is an approach that may contribute to overcome certain caveats mentioned above.

First, a higher $CRY_{n/d}$ would result from more remarkable sequential alterations between increase and decrease CRY level collected, respectively, during night and day, supposedly reflecting a closer to normal circadian rhythm. The use of this proportion instead of absolute values may diminish the influence of amount of bleeding on results.

Second, because CRY expression is ubiquitous, the cell type that serves as source of CRY in the CSF cannot be determined in this study protocol. A low $CRY_{n/d}$ may reflect low increase in CRY expression in white blood cells during night and, therefore, a lower anti-inflammatory activity. Additionally, a low $CRY_{n/d}$ may reflect a lower number of hypothalamus neurons in the same way that the level of the hypothalamus-produced hormones oxytocin and vasopressin is lower in the CSF of SAH cases with poor outcome (31). An indication whether the level of CRY in the CSF is altered due to less intense increase of CRY expression in white blood cells or due to brain damage may be revealed from the analysis of $WBCC_{n/d}$ (or alternatively of CRY expression in CSF leukocytes).

This study protocol intends to be a pilot study and the primary endpoint will be 1-year outcome defined according to modified Rankin scale as good (0–3) or poor (4–6). This option may yield a preliminary conclusion using Mann–Whitney *U*-test.

Complementary, we will analyze other functional scales, namely, Glasgow Outcome Scale and Barthel Index. Because half of the survivors after SAH displays good functional outcome measured by modified Rankin scale, but half of these patients shows neuropsychological alterations (32), we will explore this issue through the Mini-Mental Status Exam (33) and Montreal Cognitive Assessment (MoCA) (32, 34, 35). Moreover, SAH outcome studies have been taking into account analyses of health-related quality of life, which in our study will be evaluated through Sickness Impact Profile and Short-Form 36 (36, 37). Regarding timing, all outcome tests will be performed at discharge, and 1, 3, 6, and 12 months after bleeding.

## POTENTIAL IMPLICATIONS OF THE HYPOTHESIS

The confirmation of the expected results would represent one more piece of evidence that circadian rhythms parallel endogenous mechanisms of brain plasticity and can be used to predict neurologic status (**Figure 1**). Regarding the study protocol we propose here, neurologic status corresponds to intracranial hypertension during the acute phase of SAH and to functional outcome in the long term.

This confirmation may underpin therapies guided by circadian rhythm modulation. A recent study showed that hCRY2 senses magnetic field (8). Magnetic field can be manipulated in clinical practice through transcranial magnetic stimulation, which interestingly increases neurogenesis (38). Perhaps transcranial magnetic stimulation improves neurologic outcome in a series of neuropsychiatric diseases through stimulation of hCRY expression and consequent neurogenesis.

Blue light stimulation is another clinical measurement that may modulate circadian rhythm. In this regard, flashing light improves Alzheimer's disease in experimental model (39). The retina is a major structure where hCRYs are expressed (10). Blue light also acts on hCRY expression in peripheral tissues. For example, jaundice improvement in children with blue light phototherapy correlates with increase in hCRY expression in mononuclear white cells (11). Therefore, blue light interferes in the level of hCRYs through central and peripheral mechanisms, and this is the reason for which it is interesting to assess hCRY expression in CSF and white blood cells in high-grade SAH patients and the correlation of this expression with TV and $ICP_{24}$. In clinical practice, monochromatic phototherapy could follow the protocol described by Chen et al. who demonstrated CRY increase during treatment of jaundice in neonates (11). For example, in the first day of treatment, 20 W cool fluorescent bulbs could be used from 18:00 to 6:00 of the next day to reach 500 $\mu W/cm^2$ measured by a illuminance meter. This procedure is expected to increase CRY during night (which could be monitored by further CSF collections). In the following days, light exposure could be tailored aiming at reaching a $CRY_{n/d}$ that further studies occasionally reveal to be associated with good outcome.

If $CRY_{n/d}$ or $WBCC_{n/d}$ correlates with TV and $ICP_{24}$ and transcranial magnetic stimulation and blue light interfere in the level of hCRYs, then the endpoint of these therapies in high-grade SAH could also be the value of $CRY_{n/d}$ or $WBCC_{n/d}$ that leads to a TV higher than 0.666 and consequently to a predicted $ICP_{24}$ lower than 20 mmHg (**Figure 2**). Nonetheless, it is important to emphasize that these remarks are highly speculative and the practical use of these concepts depends on the confirmation of

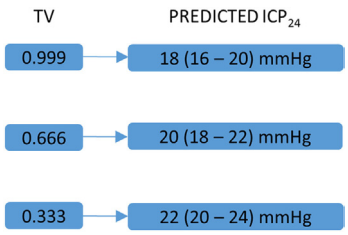


**FIGURE 2** | Key temperature variability (TV) values and corresponding predicted mean intracranial pressure in the following 24 h ($ICP_{24}$). This figure is a schematic representation of key TV values and their corresponding predicted $ICP_{24}$ [for detailed explanation, please see Ref. (2)]. Numbers between parentheses correspond to 80% prediction interval. These values could guide a further circadian rhythm modulation by stimulation of hCRY expression through transcranial magnetic and blue light stimulations.

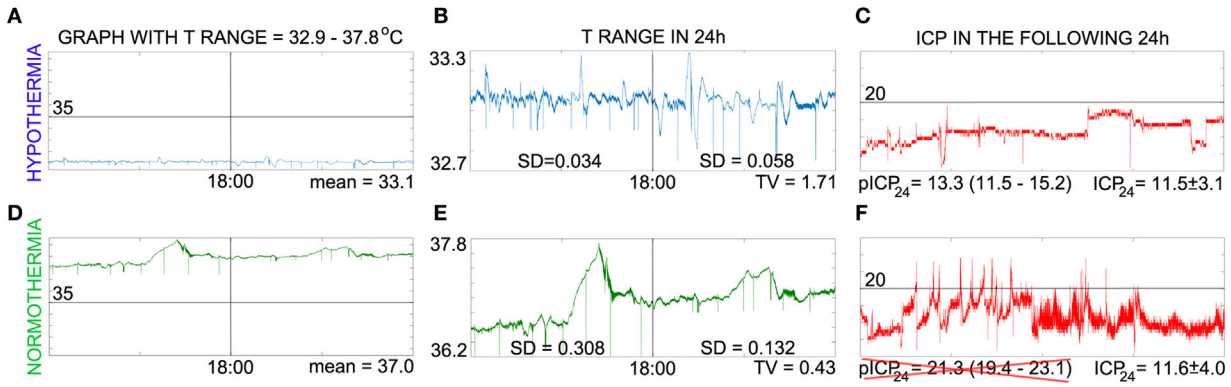


**FIGURE 3** | Caveat on intracranial pressure (ICP) prediction *via* temperature variability (TV) analysis. This panel illustrates that TV can predict ICP (in millimeter of mercury) only when mean daily T < 35°C and points to possible factor that contributes for this feature. **(A,D)** Show temperature in the same scale to visualize that absolute values were practically the same only during intravascular-induced hypothermia, which implicates that night–day ratio of coefficient of variation and night–day ratio of SD of temperature are practically the same only during hypothermia. In **(B,E)**, the temperature curves were zoomed in to show that temperature range is larger during normothermia. Note that the baseline changes in the second part of the day during normothermia [day is represented at the left side of vertical line of **(A,B,D,E)**], which reflects in the SD found; on the other hand, SD during hypothermia reflects principally temperature variation on a constant baseline. We are calculating the coefficient of variation of shorter periods (harmonics of 12 h) to see if the mean of these values can be used to derive a formula to predict $ICP_{24}$. **(C,F)** shows the ICP curves of the next day. The predicted ICP ($pICP_{24}$) matches $ICP_{24}$ only during hypothermia. For details regarding calculation, please see Ref. (2), from which **(A–C)** were adapted; **(D–F)** have not been published previously.

the hypothesis explained here, the replication of the study on ICP prediction by TV, and in the improvement of the TV analysis in such a way that it includes mean daily temperature values higher than 34.9°C (**Figure 3**). In our series, days with mean daily temperature higher than 34.9°C displayed higher temperature range. In these cases, the night–day ratio of coefficient of variation (SD/mean) was significantly different from the night–day ratio of SD, because mean night temperature over mean day temperature was not equal to one (contrarywise to what happens during hypothermia) (**Figure 3**). We are currently testing whether $ICP_{24}$ can be predicted by TV during non-hypothermia periods using variables derived from the mean coefficient of variation of harmonics of 12 h obtained during day or night, similar to the formula to predict $ICP_{24}$ already described (**Figure 3**).

## CONCLUSION

We have previously shown that the adult human brain harbors a potential neurogenic system whose integrity could be monitored by the analysis of circadian rhythms such as temperature oscillation. The pattern of circadian rhythms correlates with further brain injury, even under conditions in which the sleep–wake cycle is absent and the temperature is strictly controlled. This finding led us to postulate that light-independent mechanisms of circadian rhythm control may be involved in endogenous brain plasticity.

The circadian proteins CRYs display light-independent mechanism of circadian rhythm control, can be assessed in acute severe neurologic conditions, and their expression could theoretically be modulated by blue light and transcranial magnetic stimulation.

## AUTHOR CONTRIBUTIONS

AN (first author): developed the hypothesis and wrote the paper. AN (second author): participated in previous work of the group on the paper subject, provided ideas, and revised the manuscript. JV and MT: participated in previous work of the group on this subject, provided ideas, and revised the manuscript.

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# Time Courses of Inflammatory Markers after Aneurysmal Subarachnoid Hemorrhage and Their Possible Relevance for Future Studies

*Anke Höllig[1]*, Birgit Stoffel-Wagner[2], Hans Clusmann[1], Michael Veldeman[1], Gerrit A. Schubert[1] and Mark Coburn[3]*

*[1] Department of Neurosurgery, University Hospital RWTH Aachen, Aachen, Germany, [2] Department of Clinical Chemistry and Clinical Pharmacology, University Hospital Bonn, Bonn, Germany, [3] Department of Anesthesiology, University Hospital RWTH Aachen, Aachen, Germany*

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***Correspondence:**
*Anke Höllig*
*ahoellig@ukaachen.de*

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**Object:** Aneurysmal subarachnoid hemorrhage triggers an intense inflammatory response, which is suspected to increase the risk for secondary complications such as delayed cerebral ischemia (DCI). However, to date, the monitoring of the inflammatory response to detect secondary complications such as DCI has not become part of the clinical routine diagnostic. Here, we aim to illustrate the time courses of inflammatory parameters after aneurysmal subarachnoid hemorrhage (aSAH) and discuss the problems of inflammatory parameters as biomarkers but also their possible relevance for deeper understanding of the pathophysiology after aSAH and sophisticated planning of future studies.

**Materials and methods:** In this prospective cohort study, 109 patients with aSAH were initially included, $n = 28$ patients had to be excluded. Serum and—if possible—cerebral spinal fluid samples ($n = 48$) were retrieved at days 1, 4, 7, 10, and 14 after aSAH. Samples were analyzed for leukocyte count and C-reactive protein (CRP) (serum samples only) as well as matrix metallopeptidase 9 (MMP9), intercellular adhesion molecule 1 (ICAM1), and leukemia inhibitory factor (LIF) [both serum and cerebrospinal fluid (CSF) samples]. Time courses of the inflammatory parameters were displayed and related to the occurrence of DCI.

**Results:** We illustrate the time courses of leukocyte count, CRP, MMP9, ICAM1, and LIF in patients' serum samples from the first until the 14th day after aSAH. Time courses of MMP9, ICAM1, and LIF in CSF samples are demonstrated. Furthermore, no significant difference was shown relating the time courses to the occurrence of DCI.

**Conclusion:** We estimate that the wide range of the measured values hampers their interpretation and usage as a biomarker. However, understanding the inflammatory response after aSAH and generating a multicenter database may facilitate further studies: realistic sample size calculations on the basis of a multicenter database will increase the quality and clinical relevance of the acquired results.

**Keywords: inflammatory response, subarachnoid hemorrhage, early brain injury, delayed cerebral ischemia, observational research**

## INTRODUCTION

Early brain injury (EBI) after aneurysmal subarachnoid hemorrhage (aSAH) repeatedly has been associated with a rise of inflammatory parameters measured in serum and cerebrospinal fluid (CSF). However, results are ambiguous concerning the prognostic value of inflammatory serum or CSF parameters. Several studies have proposed single inflammatory markers to be predictive for delayed cerebral ischemia (DCI) or outcome (1–8). Thus far, due to the wide individual and interindividual range, inflammatory parameters are limited serving as biomarkers to detect complications as DCI or to predict outcome on an individual basis. However, current data are heterogeneous and partly conflicting as there is no consensus, e.g., on the compartment for sample acquisition (serum or CSF or extracellular fluid—ECF—acquired *via* microdialysis) or the time point for sampling. Neuroinflammation is a major aspect of EBI. However, it remains a challenge to differentiate beneficial from devastating inflammatory components. Furthermore, the inflammatory response to the initial injury consists of various components such as cellular reactions as in terms of microglia activation (9), induction of cytokines and chemokines (1, 8, 10), leukocyte–endothelial cell interactions (11), and modulation of receptor expression (12). A complex pattern of immunoreaction is initiated after aSAH. Besides the inflammatory activation, there are various other pathophysiological reactions, which are initiated after SAH; such as microthrombosis, cortical spreading depolarization, microvasospasm, and blood–brain barrier breakdown, altogether resulting in dysfunction of the cerebral microcirculation (13). In turn, microcirculatory dysfunction may aggravate the inflammatory activation. Thus, the interactions of the initiated reactions hamper the interpretation of a single parameter's dynamics. There is a vast amount of data on inflammatory parameters after aSAH usually in search for biomarkers predicting DCI. However, none of the examined parameters has found its way into clinical routine. This is most likely due to the abovementioned enormous individual and interindividual range and the low specificity of inflammatory parameters in general. Here, we present our data from a prospective observational study to illustrate the courses of various inflammatory parameters after aSAH. We hypothesize that monitoring of inflammatory parameters during the acute phase after aSAH does not allow an individual risk estimation (e.g., regarding the occurrence of DCI). However, detailed documentation of a vast amount of parameters may increase the understanding of the pathophysiological reaction after aSAH. Sophisticated knowledge of the pro-inflammatory reaction after aSAH and its interactions may possibly allow identification of specific anti-inflammatory therapeutic approaches.

Taken together, we aim to demonstrate that individual risk estimation for secondary complications after aSAH using inflammatory biomarkers is not promising. Instead, we vote for a multicenter database clarifying the courses and interactions of inflammatory parameters after aSAH to provide deeper insight of the pathophysiology of EBI.

## MATERIALS AND METHODS

The prospective cohort study was approved by the local ethics committee (Ethikkommission an der Medizinischen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn, Germany; EK 199/08), written informed consent according to the Declaration of Helsinki was obtained from patients or legal representatives. Data from this study have already been published elsewhere (8, 14, 15). A total of 109 consecutive patients with aSAH were screened for eligibility within a 21-month period. Patients were not eligible if they were younger than 18 years, enrolled in other clinical trials, admitted more than 12 h after onset or if informed consent could not be obtained. Due to these criteria, 28 patients were excluded ($n = 15$ declined participation or data were lost, $n = 9$ were delayed admitted, $n = 4$ participated in another trial). We documented demographic, clinical, laboratory, and radiological data within an anonymized file history. Inflammatory parameters in serum and CSF were assessed at days 1, 4, 7, 10, and 14 after aSAH. CSF was tested in patients with necessity for CSF drainage mostly due to hydrocephalus ($n = 48$). Sample acquisition regularly took place in line with routine diagnostic (between 5:00 and 6:00 a.m.).

### Sample Processing

Blood and CSF samples were centrifuged for 10 min at $2{,}000 \times g$ before processing. Samples were analyzed at the Department of Clinical Chemistry and Clinical Pharmacology, University of Bonn, either as part of routine diagnostics [leukocyte count (G/L), determination of C-reactive protein (CRP, mg/L), and interleukin 6 (IL6, pg/mL)] or for study purposes, by means of enzyme-linked immunofluorescence assays for determination matrix metallopeptidase 9 (MMP9, ng/mL), intercellular adhesion molecule 1 (ICAM1, ng/mL), and leukemia inhibitory factor (LIF, pg/mL) in serum samples (all assays purchased by IBL International GmbH, Hamburg, Germany).

### Analysis

Courses of serum parameters (all patients included), additionally CSF parameters and the corresponding serum parameters of the patients with CSF drainage were plotted dependent on time of sample acquisition. Graphs were acquired using GraphPad Prism® 6. Furthermore, courses were analyzed using the Mann–Whitney *U* test. Occurrence of DCI was used as grouping variable to illustrate the usual approach in search of a biomarker indicating the occurrence of DCI. Therefore, DCI was defined as secondary neurologic worsening (increase in modified National Institutes of Health Stroke Scale >2 points). Furthermore, clinical improvement after induced hypertension and new cerebral ischemia or perfusion deficit confirmed by cranial computed tomography or magnetic resonance imaging (in case of consciousness or sedation or persistent neurologic deficit) were defined as indicators of DCI (if not explained by other causes such as embolism during angiography). Other relevant known causes such as infection, seizure, and metabolic, or electrolyte disturbances were excluded before determination of DCI. Tests were carried out using SPSS® 21.

## RESULTS

Baseline characteristics of the patients included is presented as supplemental data table (see Table S1 in Supplementary Material). Infectious complications occurred regularly with $n = 8$ (9.9%) at day 1, $n = 27$ (33.3%) at day 4, $n = 38$ (46.9%) at day 7, $n = 36$ (44.4%) at day 10, and $n = 26$ (32.1%) at day 14.

### Interleukin 6

Serum course of IL6 differs from the pattern observed in CSF samples (**Figures 1B,C**). The peak of the measured serum level represents values obtained at day 1 after aSAH, whereas course of IL6 in CSF peaks somewhat later (at day 4) (**Figure 1A**). CSF values are far higher than serum levels (up to 10-fold). Of note, confidence intervals especially for serum values are huge due to the enormous variability of individual values.

### Matrix Metallopeptidase 9

Matrix metallopeptidase 9 peaks earlier in CSF samples (at day 4) than in serum samples (at day 7) (**Figure 2**). Contrary to IL6, MMP9 is found in patients' serum in far higher concentrations then in CSF.

### Intercellular Adhesion Molecule 1

Intercellular adhesion molecule 1 is detectable in all of the acquired serum samples. In CSF samples, lower limit of detection (6.3 ng/mL) was not reached in 45.8% ($n = 22$) of the samples. Highest value of CSF ICAM1 expression was 56.8 ng/mL. Of note, the peak of the course is observed at day 4 after aSAH (**Figure 3**). However, variability especially at day 4 is extraordinary high.

### Leukemia Inhibitory Factor

Leukemia inhibitory factor was detected reliably in patients' CSF samples (LOD: 3.13 pg/mL). The course of LIF measured in CSF differs a lot from the one measured in serum samples (**Figure 4**): in CSF, a very early peak is observed followed by a rapid drop (**Figure 4A**). The assessed CSF levels are quite homogeneous demonstrated by low SDs. Serum levels of LIF are far lower (up to 10-fold) (**Figures 4B,C**).

### CRP and Leukocyte Count

The levels of CRP and leukocyte count were only assessed in serum samples. Of note, courses of CRP and leukocyte count act almost inversely. At day 4 after aSAH CRP levels peaks (**Figure 5**), whereas leukocyte count shows its nadir (**Figure 6**).

### Courses of Inflammatory Parameters Related to DCI

In 18 patients (22.2%), occurrence of DCI was detected (day 3: $n = 1$; day 4: $n = 5$; day 6: $n = 2$; day 7: $n = 8$; day 9: $n = 1$; day 10: $n = 1$). Comparing the courses of the assessed parameters related

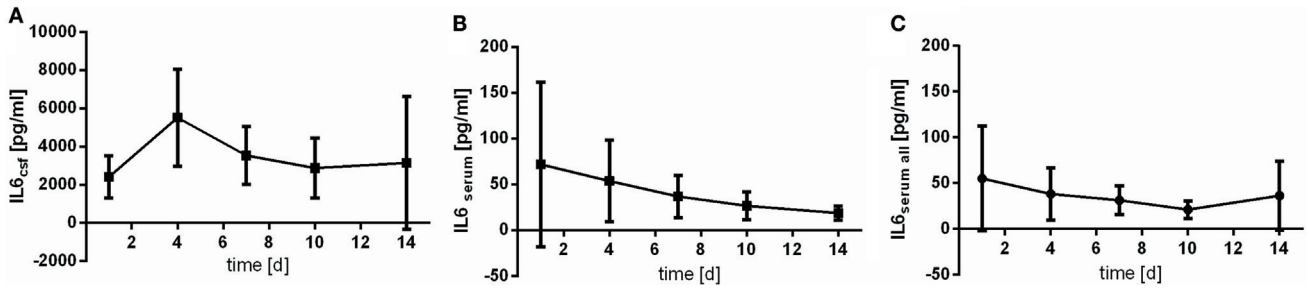


**FIGURE 1** | Time courses of interleukin 6 (IL6) in cerebrospinal fluid (CSF) samples [**(A)**; IL6csf], serum samples of the patients with CSF samples [**(B)**; IL6serum], and all serum samples available [**(C)**; IL6serum all] and their 95% confidence interval are displayed. Differences between nadir and peak values assessed *via* Wilcoxon Rank Test: IL6serum: day 14 vs. day 1: $p = 0.029$; IL6csf: day 1 vs. day 4: $p = 0.184$.

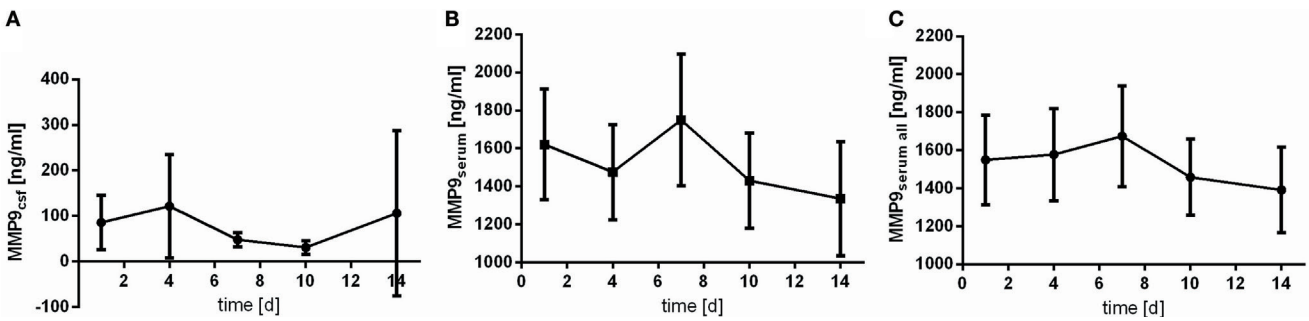


**FIGURE 2** | Time courses of matrix metallopeptidase 9 (MMP9) in cerebrospinal fluid (CSF) samples [**(A)**; MMP9csf], serum samples of the patients with CSF samples [**(B)**; MMP9serum], and all serum samples available [**(C)**; MMP9serum all] and their 95% confidence interval are displayed. Differences between nadir and peak values assessed *via* Wilcoxon Rank Test: MMP9serum: day 14 vs. day 7: $p = 0.103$; MMP9csf: day 10 vs. day 4: $p = 0.001$.

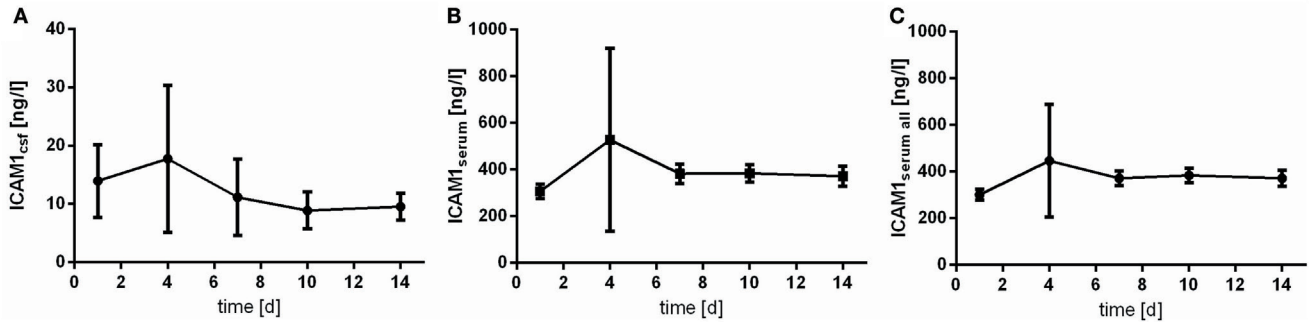


**FIGURE 3** | Time courses of intercellular adhesion molecule 1 (ICAM1) in cerebrospinal fluid (CSF) samples [**(A)**; ICAM1csf], serum samples of the patients with CSF samples [**(B)**; ICAM1serum], and all serum samples available [**(C)**; ICAM1serum all] and their 95% confidence interval are displayed. Differences between nadir and peak values assessed *via* Wilcoxon Rank Test: ICAMserum: day 1 vs. day 4: $p = 0.001$; IL6csf: day 10 vs. day 4: $p = 0.906$.

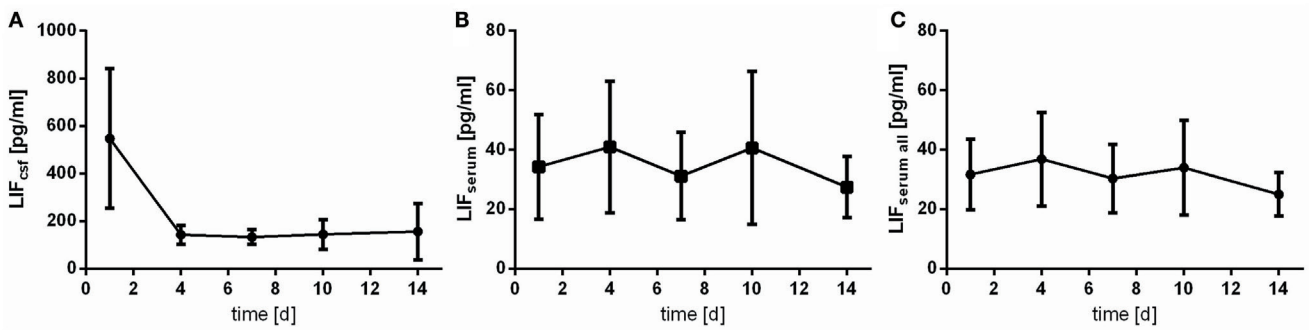


**FIGURE 4** | Time courses of leukemia inhibitory factor (LIF) in cerebrospinal fluid (CSF) samples [**(A)**; LIFcsf], serum samples of the patients with CSF samples [**(B)**; LIFserum], and all serum samples available [**(C)**; LIFserum all] and their 95% confidence interval are displayed. Differences between nadir and peak values assessed *via* Wilcoxon Rank Test: LIFserum: day 14 vs. day 4: $p = 0.081$; IL6csf: day 4 vs. day 1: $p < 0.0001$.

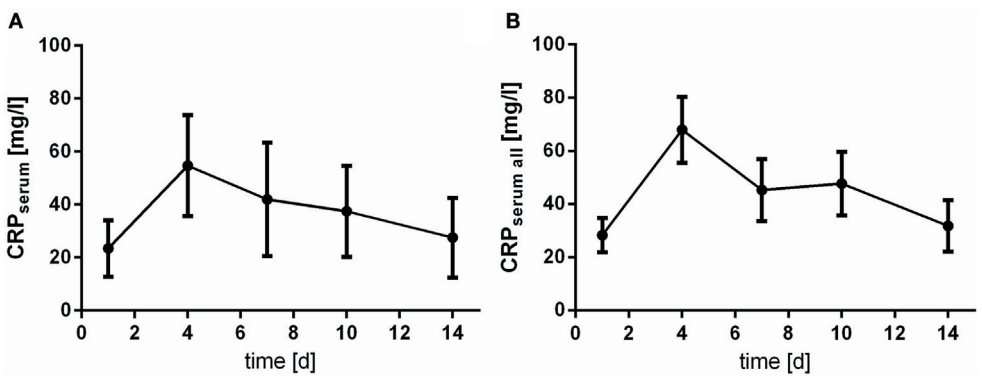


**FIGURE 5** | Time courses of C-reactive protein (CRP) in serum samples of the patients with cerebrospinal fluid samples [**(A)**; CRPserum] and all serum samples available [**(B)**; CRPserum all] and their 95% confidence interval are displayed. Differences between nadir and peak values assessed *via* Wilcoxon Rank Test: CRP: day 1 vs. day 4: $p < 0.0001$.

to occurrence of DCI, no significant differences were detected (Mann–Whitney *U* test). Marginal significance was only seen for leukocyte count at day 4 ($p = 0.054$). Courses are provided in **Figure 7**.

## Courses of Inflammatory Parameters Related to Fisher Scale Score

Courses of inflammatory parameters additionally were compared related to Fisher scale score. Grading according to Fisher scale score

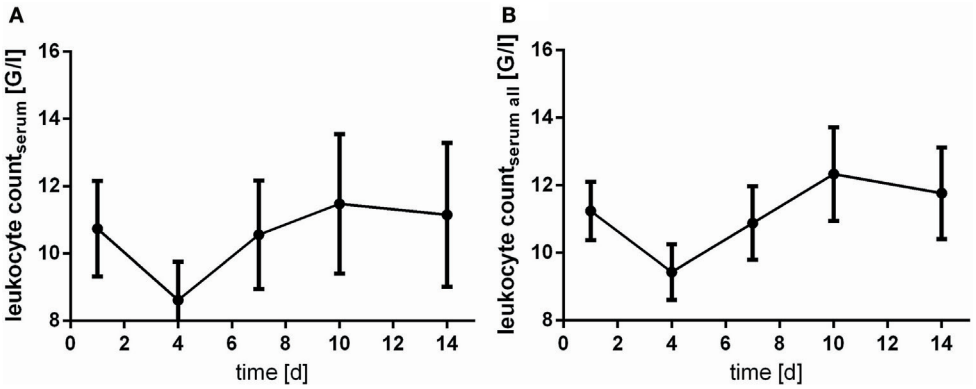


**FIGURE 6** | Time courses of leukocyte count in serum samples of the patients with cerebrospinal fluid samples [**(A)**; leukocyte countserum] and all serum samples available [**(B)**; leukocyte countserum all] and their 95% confidence interval are displayed. Differences between nadir and peak values assessed *via* Wilcoxon Rank Test: leukocyte count: day 4 vs. day 10: $p < 0.0001$.

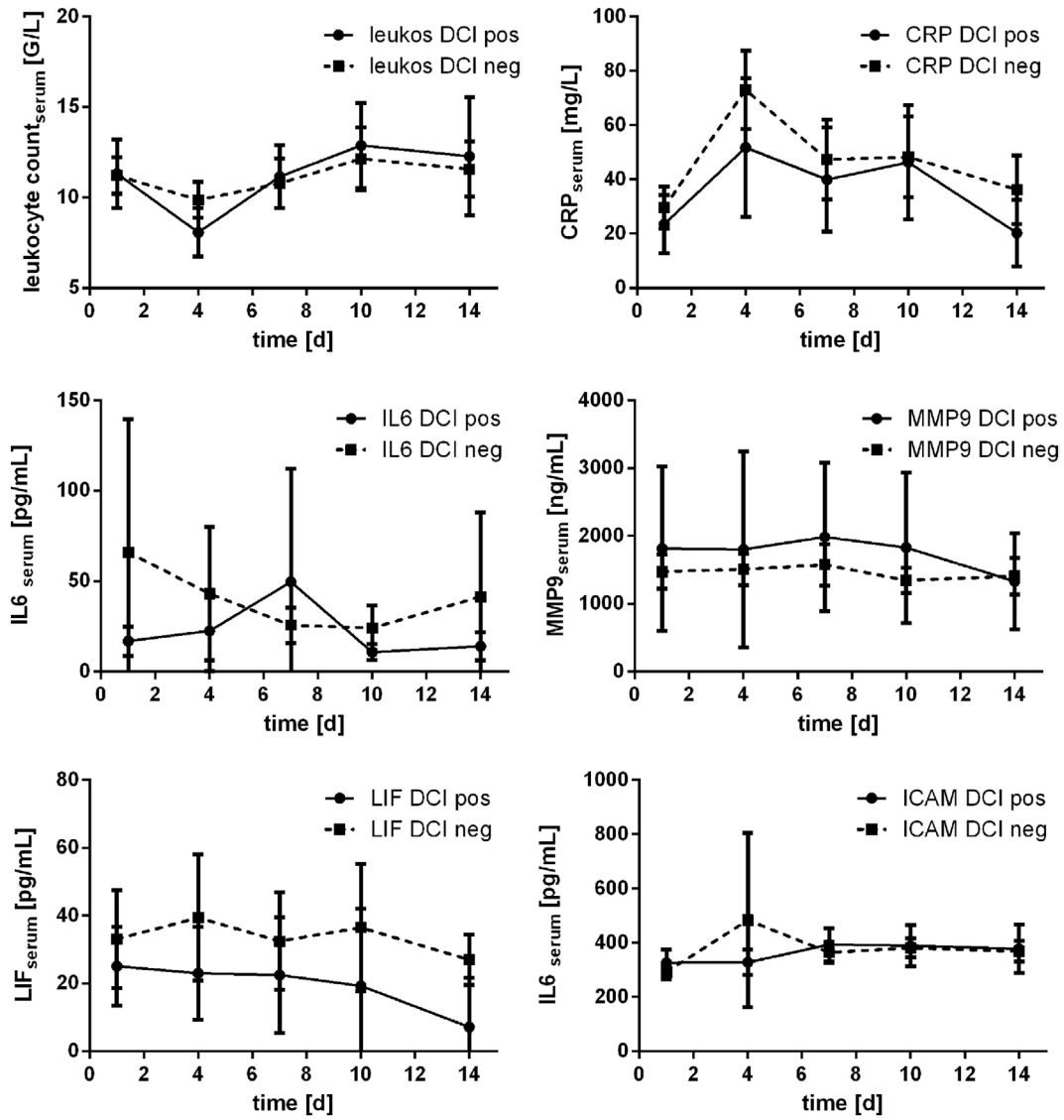


**FIGURE 7** | Courses of serum inflammatory parameters related to occurrence of delayed cerebral ischemia (DCI) (±95% confidence interval).

was available for $n = 81$ patients [Fisher 1: $n = 1$ (1.2%); Fisher 2: $n = 13$ (16%); Fisher 3: $n = 27$ (33.3%); and Fisher 4: $n = 40$ (49.4%)]. For statistical purposes, Fisher grading was dichotomized according to severity of aSAH (Fisher A: Fisher 1 and 2; Fisher B: Fisher 3 and 4). Especially course of CRP was related to Fisher grading: the measured values significantly differed related to dichotomized Fisher scale score at day 1 ($p = 0.019$), day 4 ($p = 0.001$), and day 7 ($p = 0.033$; Mann–Whitney $U$ test). Significant difference was also seen for serum IL6 at day 1 ($p = 0.006$) and day 4 ($p = 0.002$), further for serum LIF at day 7 ($p = 0.038$; Mann–Whitney $U$ test). All courses are displayed in **Figure 8**.

## Courses of Inflammatory Parameters Related to WFNS Scale Score

Courses of inflammatory parameters also have been related to the impact of hemorrhage expressed according to WFNS scale score, which may reflect the EBI. The score was available for $n = 81$ patients [WFNS 1: $n = 24$ (29.6%); WFNS 2: $n = 15$ (18.5%); WFNS 3: $n = 5$ (6.2%); WFNS 4: $n = 14$ (7.3%); WFNS 5: $n = 23$ (28.5%)]. Again, grading scale scores were dichotomized for statistical purposes (WFNS A: WFNS 1–3; WFNS B: WFNS 4 and 5). Particularly courses of IL6 levels were related to the impact of hemorrhage expressed according to WFNS scale score: significant differences of the values for the WFNS A vs. WFNS B group were found at day 1 ($p = 0.001$); day 7 ($p = 0.007$); day 10 ($p = 0.010$), and day 14 ($p = 0.035$; Mann–Whitney $U$ test). From day 1 to 10, mean IL6 values in the WFNS A group were lower than in the WFNS B group, but at day 14 it changed to higher mean values in the WFNS A group compared with WFNS B.

Levels of CRP measured early after the impact of aSAH also were found to be related to WFNS scale score: at days 1 and 4 significantly higher values were seen in the WFNS B group

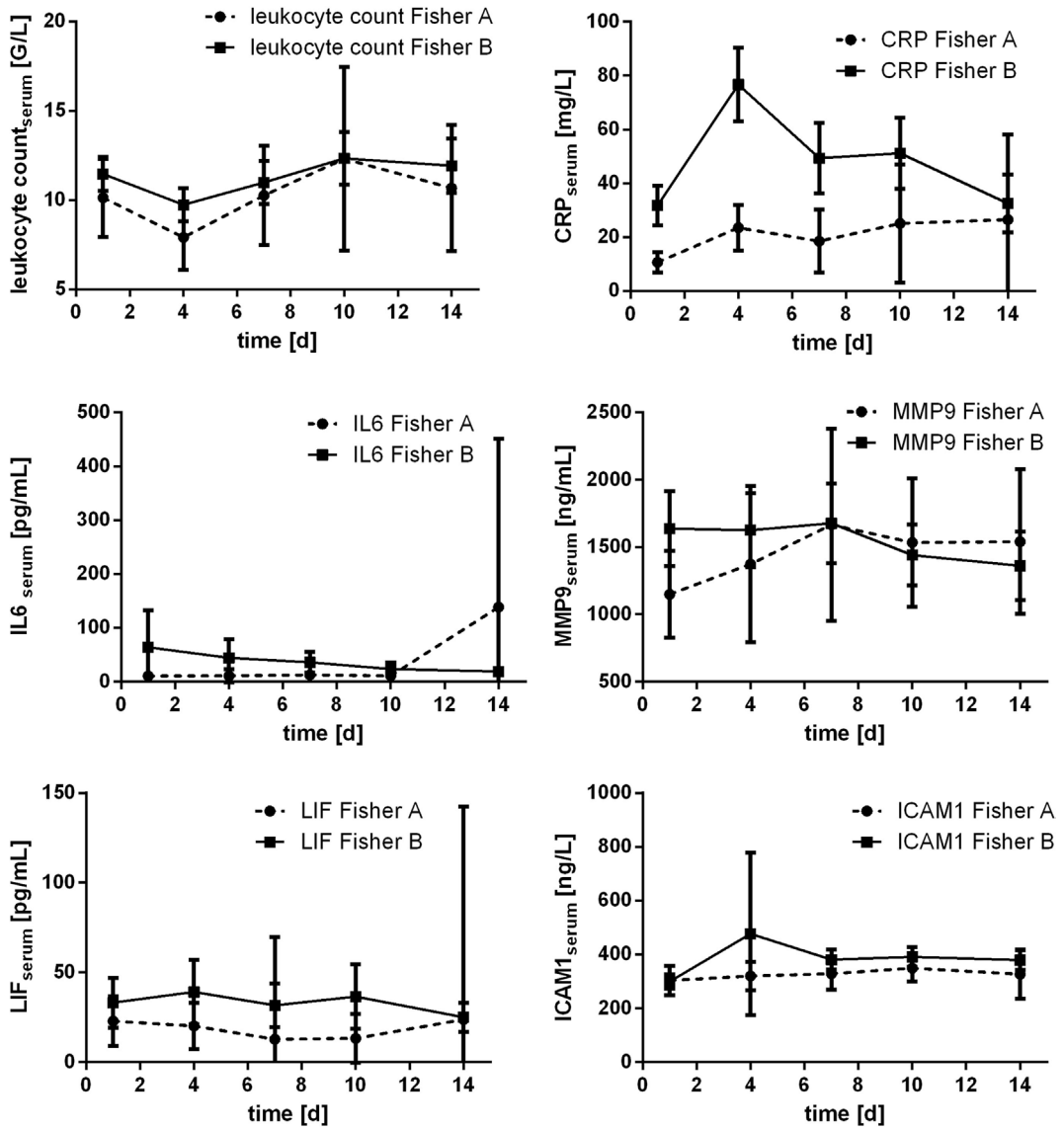


**FIGURE 8** | Courses of serum inflammatory parameters related to dichotomized Fisher scale score (±95% confidence interval).

compared with the WFNS A group (day 1: $p = 0.039$; day 4: $p = 0.034$; Mann–Whitney $U$ test).

No significant differences related to dichotomized WFNS scale score were found for leukocyte count, MMP9, LIF, and ICAM.

All courses are displayed in **Figure 9**.

## DISCUSSION

Here, we illustrate the courses of several inflammatory parameters (IL6, MMP9, ICAM1, and LIF) in CSF and serum samples after aSAH, additionally courses of CRP and leukocyte count in serum. The levels of IL6 and LIF are far higher in CSF samples than in serum samples during the entire observation period, whereas MMP9 and ICAM1 were detected in higher concentrations in serum samples. The courses of CRP and leukocyte count nearly act inversely.

Our evaluation of the measured values related to the occurrence of DCI illustrated the usual problem that the wide range of the measured values hampers their interpretation and usage as a biomarker. However, understanding the inflammatory response after aSAH and generating a database may facilitate further studies: realistic sample size calculations on the basis of a multicenter database will increase the quality and clinical relevance of the acquired results.

Interleukin 6 is a pleiotropic cytokine. Its secretion is induced by various stimuli (such as infectious disease, trauma, and other causes of tissue damage, but also during chronic conditions, e.g., depression or chronic pain). There are plenty of publications dealing with the influence of IL6 secretion after aSAH on outcome and occurrence of DCI (1, 2, 8, 10, 14, 16–19). Time courses of cytokine levels in CSF provided by us are similar to those illustrated by Niwa and colleagues (10), However, others

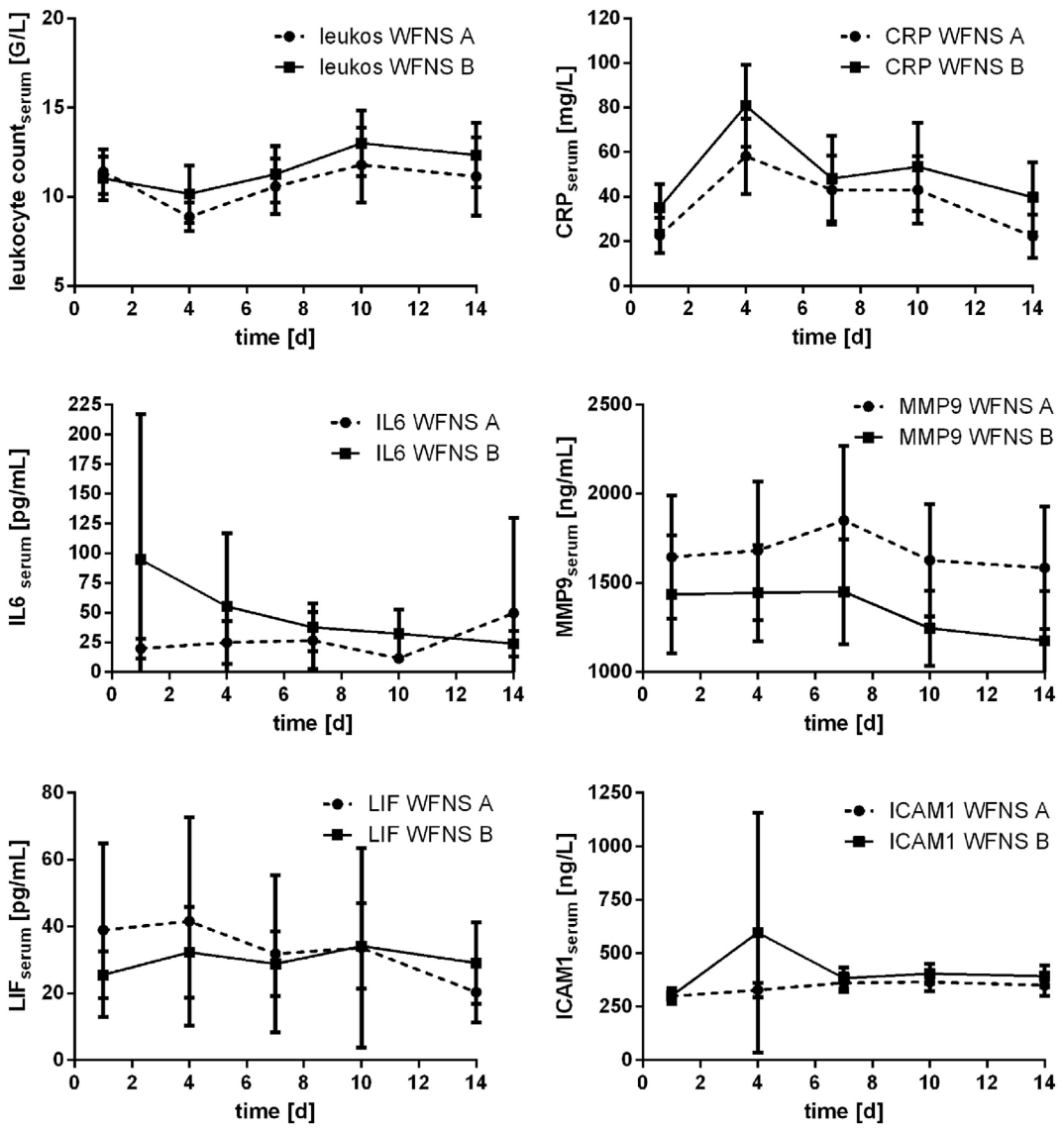


**FIGURE 9** | Courses of serum inflammatory parameters related to dichotomized WFNS scale score (±95% confidence interval).

demonstrate a later peak (1, 20). In total, schedules for sampling acquisition differ a lot among the previously mentioned studies; thus, comparison of results or courses is hampered. Anyway, wide ranges of IL6 levels are seen in all of the studies. Of note, related to WFNS scale score IL6 levels were found to be indicators for injury's severity in our study. Nevertheless, the usage of IL6 as a biomarker is hampered by its non-specificity and its ambiguous effect showing both pro-inflammatory and destructive as well as regenerative aspects.

Higher levels of MMP9 have been associated with unfavorable outcome (21). Similar to our data, SDs (i.e., variance of measured values) are pronounced, and serum levels are more than 10-fold higher than the CSF levels.

The interpretation of ICAM1 in CSF samples is hampered by the LOD of the used assay, as the LOD was not reached in 45.8% of the samples. The ICAM1 CSF levels in our cohort are somewhat lower than those presented by Kaynar and colleagues (22), serum levels are similar. Nevertheless, comparison of time course is not possible due to different schedule for sample acquisition. Mack and colleagues also have presented ICAM1 serum levels over time after aSAH (23). Time courses seem to be similar; however, serum levels measured are slightly lower.

There are no previous data on LIF after aSAH. We demonstrate an early peak in CSF samples with far higher overall levels than in serum samples.

Leukocyte count and CRP levels have been associated with prognosis as well as vulnerability for DCI (5, 21, 24–27). Both parameters are unspecific and therefore not useful as biomarkers. Anyway, pronounced leukocytosis seems to reflect severity of disease and consecutively prognosis.

We present time courses of CSF and serum inflammatory parameters after aSAH. The results of the study are limited by the fact that time courses are displayed only descriptively. Furthermore, sample size of the presented data is quite low.

However, we estimate that mere description is useful to understand the complex inflammatory response after aSAH.

Due to their marginal specificity and wide interindividual range, inflammatory parameters hardly serve as biomarkers. Nevertheless, there is a distinct inflammatory response after aSAH, which includes a vast spectrum of parameters interacting with each other. Beneficial as well as damaging stimuli may result from the inflammatory reaction. The interactions of this reaction are poorly understood. As far as our experience goes, we hold that further illustration of courses and possible interactions of inflammatory reaction after aSAH is essential before associations with outcome or occurrence of DCI in search for a biomarker are promising. Generally, setting up multicenter databases to further illustrate and hopefully understand inflammatory response after aSAH has more prospect of success than searching for a single biomarker resulting in controversial, clinically irrelevant results. Furthermore, these data may lay the groundwork for further studies' realistic sample size calculations to create clinically relevant and reliable results. Thus, we encourage everyone involved in research of inflammation after aSAH to cooperate and build up networks to share acquired data.

## ETHICS STATEMENT

This study was carried out in accordance with the recommendations of "Ethikkommission an der Medizinischen Fakultät der Rheinischen Friedrich-Wilhelms Universität Bonn name of guidelines, name of committee" with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the local ethics committee (Ethikkommission an der Medizinischen Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn, Germany; EK 199/08).

## AUTHOR CONTRIBUTIONS

AH conceived the study, analyzed the data, and wrote the preliminary draft. BS-W organized and supervised analysis of serum and CSF samples; assisted in interpretation of data. MV and HC participated in the analyses and helped to draft the manuscript. GS helped with data analyses and assisted the revision of the manuscript. MC participated in the design of the study and coordination and helped to draft the manuscript. All the authors read, revised, and approved the final manuscript.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/articles/10.3389/fneur.2017.00694/full#supplementary-material.

**TABLE S1** | Baseline characteristics of patients included.

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# Heparin: The Silver Bullet of Aneurysmal Subarachnoid Hemorrhage?

*Nicolas K. Khattar and Robert F. James**

*Department of Neurological Surgery, University of Louisville School of Medicine, Louisville, KY, United States*

Various neurological diseases have recently been associated with neuroinflammation and worsening outcomes. Subarachnoid hemorrhage has been shown to generate a potent neuroinflammatory response. Heparin is a potential effective anti-inflammatory agent to prevent initial injury as well as delayed neurological decline. Different mechanisms of action for heparin have been proposed including, but not limited to the binding and neutralization of oxyhemoglobin, decreased transcription and signal transduction of endothelin-1, inhibition of binding to vessel wall selectins and vascular leakage into the subarachnoid space as well as direct binding and neutralization of inflammatory molecules. With a reasonably safe side-effect profile, heparin has shown significant promise in small series in human studies of aneurysmal subarachnoid hemorrhage in decreasing both initial and delayed neurological injury. Further studies are needed to validate various neuroprotective features of heparin in subarachnoid hemorrhage as well as other disease states.

**Keywords: unfractionated heparin, neuroprotection, neuroinflammation, subarachnoid hemorrhage, delayed neurological injury, vasospasm**

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***Correspondence:**
*Robert F. James*
*robert.james@louisville.edu*

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## INTRODUCTION

Neuroinflammation has been recently associated with worse functional outcomes in patients afflicted by a plethora of neurological diseases, but especially in aneurysmal subarachnoid hemorrhage (aSAH) (1). Inflammation has been designated as a primary cytotoxic event affecting neurons in both acute and chronic neurological diseases. This has led to specific targeting of the inflammatory cascade (2). Unfractionated heparin (UFH) is a safe and commonly used anticoagulant in the treatment of deep venous thrombosis, pulmonary emboli, and other hypercoagulable conditions. In addition to its anticoagulant properties, UFH has been shown to have very promising significant neuroprotective anti-inflammatory properties, especially in association with subarachnoid hemorrhage (3–5). Studies have shown that heparin is prevalent in various invertebrates with no hematological system, indicating that its primary physiological role is not anticoagulation (6). Even though extrapolation to other species is not evident, the role of heparin is worth investigating. In this short review, we focus on the ever-expanding connection between heparin and neuroprotection in the setting of aSHA.

## PROFILE: CHARACTERISTICS AND MECHANISM OF ACTION

Unfractionated heparin is a highly sulfated glycosaminoglycan polymer with the most negative charge of any biological molecule (7). Inflammatory mediators are generally positively charged and binding to the heparin molecule disturbs the biochemical and electrostatic microenvironment, effectively neutralizing the actions of these mediators (8). Particularly in hemorrhagic stroke, heparin is able

to bind to oxyhemoglobin and neutralize the toxic effects of free hemoglobin on other structures in the brain (9). Given the large size and uniform distribution of the negative charge along the protein, heparin can stoichiometrically bind four molecules of oxyhemoglobin, thereby quickly neutralizing the noxious effects of the oxygen free radicals on the endothelium as well as the brain parenchyma (9).

On the molecular level, heparin was found to have various neuroprotective interactions. It is able to decrease the transcription of endothelin-1 (ET-1) and the ET-1 promoter. In addition, heparin decreased the expression of the erythroid transcription factor family (GATA)-binding capacity, which is essential for ET-1 function in the endothelial cells (10, 11). ET-1 displays significant vasoconstrictive effects mediated in vascular smooth muscle cells through the epidermal growth factor receptor (EGFR) (12). Heparin-binding epidermal growth factor, a ligand of EGFR, modulates its transactivation (12). Binding of heparin to the ligand prevents EGFR receptor transactivation and was found to dampen any peri-hemorrhagic vasoconstriction and further cerebral injury in both murine and human *in vitro* models (12, 13).

Heparin and its low-molecular weight derivatives are potent inhibitors of the adhesion molecules P- and L-selectin, which mediate leukocyte rolling, the initial event governing leukocyte transmigration from vessel walls into areas of inflammation (14, 15). These mechanisms mirror effects observed in cancer metastasis, where UFH (and not other anticoagulants) was able to decrease the speed of oncologic spread by inhibiting the selectins (15). This particular effect has been further explored in cancer research and has been shown to be potent with any type of adhesion molecules (galectins, integrins, etc.), in addition to being shown to be completely unrelated to the anticoagulant properties of heparin (16). All effects were preserved with modified 2- or 6-O desulfated, N-acetylated heparins in both the oncologic and neurovascular models (16, 17). Heparin was also found to bind all the pro-inflammatory molecules such as cytokines, chemokines, as well as mediators of inflammation such as elastase and major basic protein (18–20).

Translocation of inflammatory leukocytes into an injured area through vascular leakage is a hallmark of the initiation of the inflammatory response. Presence of leukocytes in the subarachnoid space is a specific marker of inflammation (21). The accepted mechanism of action of leukocyte extravasation is through interaction of cell glycoproteins with the selectin family of proteins, generally expressed on endothelial cells (22). Heparin has been shown to specifically decrease the number of leukocytes participating in the inflammatory response to any insult to the central nervous system (23, 24).

Prior studies have also shown that microthromboembolisms may have contributed to delayed neurological deficits, therefore possibly implicating the anticoagulation effect of heparin in the neuroprotective effects observed (25). This hypothesis has been demonstrated in experimental animal models of subarachnoid hemorrhage. Mice in the experimental group showed an increase in the number of microthrombi when compared with the sham group (26). On the other hand, the advent of modifications of heparin lacking the anticoagulation domains and showing anti-inflammatory neuroprotection provides some evidence to suggest microthromboemolisms do not represent the entire pathological picture of aSHA (27). A head-to-head comparison of both forms of heparin will need to be conducted to elucidate the roles of the various domains of heparin. The various proposed mechanisms of action of UFH are summarized in **Table 1**.

## NEUROPROTECTIVE EFFECTS OF HEPARIN IN SUBARACHNOID HEMORRHAGE

Some authors hypothesize that a combination of the hemorrhage volume, vasoparalysis, and decreased cerebrospinal fluid resorption associated with aSHA triggers an increase in intracranial pressure leading to significant transient ischemia. This has been considered by various authors to be the cause of the initial neurological injury following the ictus, especially when associated with loss of consciousness at presentation (28, 29). Heparin and its non-anticoagulant derivatives have been shown to effectively counter the initial injury in various rat models of ischemia/reperfusion mimicking the initial insult following aneurysmal rupture (27, 30). The caspase pathway is triggered in the initial phase of the injury of subarachnoid hemorrhage, especially given that activation of caspase 1 was first observed in ischemic stroke (31). Countering the early activation of caspases 1, 3, 8, 9, and 11 in global cerebral ischemia can prevent neurological devastation. Animal studies demonstrated a significant decrease in cleaved caspase 3 in samples obtained from subarachnoid hemorrhage animals that were

**TABLE 1** | Mechanisms of action of heparin to prevent delayed neurological injury associated with subarachnoid hemorrhage.

*Direct chelation of hemoglobin in the subarachnoid space*
- Heparin binds oxidized hemoglobin that is released from damaged erythrocytes. Oxyhemoglobin is believed to have a major role in the induction of vasospasm

*Decreased free-radical release*
- Heparin is able to directly bind to specific molecules and inhibit the formation of free radicals through the inhibition of various pro-inflammatory molecules that contribute to their formation

*Inhibition of endothelin-1 (ET-1)*
- Inhibition of mRNA transcription of ET-1
- Inhibition of transactivation of the epidermal growth factor receptor by binding of heparin to the specific ligand
- Suppression of release of intracellular calcium and inositol-triphosphate in addition to ET-1 release
- Inhibition of MAP-K and prevention of DNA synthesis induced by ET-1

*Prevention of $K^+$ channel down-regulation induced by oxyhemoglobin release*
- Downregulation of potassium channels causes a depolarization of vascular smooth muscle cells, increased incidence of calcium influxes and increased activation causing increased vasoconstriction and neurological decline

*Suppression of vascular smooth cell hyperplasia*
- Smooth muscle and myofibroblast proliferation, associated with cell necrosis, lead to increased vasoconstriction, ischemia, and further neurological decline. Pathological proliferation of smooth muscle cells and neovascularization may prevent further progression of neurological injury

*Inhibition of neuroinflammatory pathways*
- Inhibition of the NF-kB pathway
- Binding of chemokines, cytokines, and other inflammatory proteins

given heparin pharmacotherapy. The downregulation of apoptotic effectors leads to decreased neuroinflammation, demyelination, and decreased burden of injury (32).

The evidence supporting heparin's anti-inflammatory role is further supported with evidence from traumatic brain injury (TBI) and chronic neurodegeneration research. Nagata et al. has shown that early administration of heparin is associated with a significant decrease in post-TBI inflammation and preservation of cognitive outcomes. These results were further supported by prior studies investigating the role of low-molecular weight fractionated heparin, enoxaparin (2, 23, 33–35). Human studies investigated the role of enoxaparin (low-molecular weight fractionated heparin) in aneurysmal SAH with mixed results (36, 37). There was a statistically significant reduction in delayed ischemia and vasospasm in the enoxaparin group in one trial but no obvious benefit in others (36, 37). A retrospective cohort study showed significant benefits of UFH in Fisher grade 3 subarachnoid hemorrhage patients (4). The patients in the heparin group had significantly less clinical and radiographic vasospasm, as well as a decrease in vasospasm-related infarction. In addition, there were a significantly higher proportion of patients who were discharged home from the hospital, instead of having to be discharged to a rehabilitation facility (4). Human studies highlighting the neuroprotective effects of heparin in various disease states are summarized in **Table 2**.

In addition to the transient ischemia state associated with SAH, significant vasogenic edema and blood–brain barrier dysfunction plague this patient population and represent a dismal prognostic factor (39). Heparin has been shown to counter cerebral edema in general in various pathological states including but not limited to TBI, meningitis, ischemia, and intracerebral hemorrhages (23, 40–43). Various interactions in animal models have been reported between heparin and molecules specifically associated with cerebral edema (VEGF, bradykinin, etc.) but no proven connection linking the action of heparin on these molecules was proven.

These neuroprotective effects have been shown to correlate with a decrease in clinical vasospasm and a specific decrease in the delayed neurological injury, namely long-term cognitive decline following subarachnoid hemorrhage (32, 44, 45). Despite the fact that the number of patients included in these series remains limited; there is a positive trend and a budding interest in a closer correlation of heparin with improved cognitive outcomes. One such effort is the Aneurysmal Subarachnoid hemorrhage Trial RandOmizing Heparin, a Phase II multi-center randomized trial, studying the effects of low-dose intravenous heparin infusion on 90-day cognitive outcomes using the Montreal Cognitive Assessment test. Enrollment is expected to be complete in December 2018 (NCT02501434).

**TABLE 2** | Human studies showing neuroprotection of heparin in various neurological injuries.

| |
|---|
| *Prevention of delayed neurological injury following aneurysmal subarachnoid hemorrhage* |
| – Low-dose IV heparin has been associated with a decrease in the rate of cerebral vasospasm (4) |
| – Low-dose IV heparin may be associated with improved cognitive outcomes and a decrease in delayed neurological deficits (38) |
| *Prevention of neurological sequelae following traumatic brain injury (TBI)* |
| – Early initiation of heparin therapy in TBI patients is associated with no neurological deterioration and decreased progression of injury on imaging (34) |
| *Prevention of metastasis in neoplasia* |
| – Heparin has been associated with decrease/delay of metastasis in various cancers due to prevention of blood–brain barrier breakdown and spread of monoclonal cells into the central nervous system space (15) |

## CONCLUSION AND FUTURE DIRECTIONS

Heparin is today a venerable, ever-young drug that has not ceased to bewilder and amaze. With its seemingly ubiquitous properties, heparin seems to show promise in various neuropathologies, with a predictable and manageable side-effect profile. Further research is needed to establish heparin as a completely safe and effective intervention in patients with subarachnoid hemorrhage. Together, with its success in other experimental neurological insults such as TBI, stroke, meningitis, cancer, etc., heparin seems to be emerging as a potential silver bullet for mitigating the delayed neurological injury commonly seen after aSHA.

## AUTHOR CONTRIBUTIONS

RJ designed the concept of the review. NK conducted the review, compiled the information, and drafted the initial manuscript. Both authors critically reviewed and approved the final version of the manuscript.

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REVIEW
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# Neuroinflammation as a Target for Intervention in Subarachnoid Hemorrhage

*Airton Leonardo de Oliveira Manoel*[1,2]* *and R. Loch Macdonald*[3]

[1] *Adult Critical Care Unit, Hospital Paulistano – United Health Group, São Paulo, Brazil,* [2] *Keenan Research Center for Biomedical Science, Department of Surgery, Li Ka Shing Knowledge Institute, University of Toronto, Toronto, ON, Canada,* [3] *Division of Neurosurgery, St. Michael's Hospital, Labatt Family Centre of Excellence in Brain Injury and Trauma Research, Keenan Research Centre for Biomedical Science, Department of Surgery, Li Ka Shing Knowledge Institute, University of Toronto, Toronto, ON, Canada*

Aneurysmal subarachnoid hemorrhage (SAH) is a sub-type of hemorrhagic stroke associated with the highest rates of mortality and long-term neurological disabilities. Despite the improvement in the management of SAH patients and the reduction in case fatality in the last decades, disability and mortality remain high in this population. Brain injury can occur immediately and in the first days after SAH. This early brain injury can be due to physical effects on the brain such as increased intracranial pressure, herniations, intracerebral, intraventricular hemorrhage, and hydrocephalus. After the first 3 days, angiographic cerebral vasospasm (ACV) is a common neurological complication that in severe cases can lead to delayed cerebral ischemia and cerebral infarction. Consequently, the prevention and treatment of ACV continue to be a major goal. However, most treatments for ACV are vasodilators since ACV is due to arterial vasoconstriction. Other targets also have included those directed at the underlying biochemical mechanisms of brain injury such as inflammation and either independently or as a consequence, cerebral microthrombosis, cortical spreading ischemia, blood–brain barrier breakdown, and cerebral ischemia. Unfortunately, no pharmacologic treatment directed at these processes has yet shown efficacy in SAH. Enteral nimodipine and the endovascular treatment of the culprit aneurysm, remain the only treatment options supported by evidence from randomized clinical trials to improve patients' outcome. Currently, there is no intervention directly developed and approved to target neuroinflammation after SAH. The goal of this review is to provide an overview on anti-inflammatory drugs tested after aneurysmal SAH.

**Keywords: neuroinflammation, subarachnoid hemorrhage, early brain injury, secondary brain injury, vasospasm, delayed cerebral ischemia**

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***Correspondence:**
*Airton Leonardo de Oliveira Manoel*
*airtonleo.manoel@gmail.com*

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## INTRODUCTION

Aneurysmal subarachnoid hemorrhage (SAH) is a complex cerebrovascular disease with profound systemic complications (1–3). Despite the advances achieved in the management of those patients, high disability and mortality rates continue to devastate this patients' population (4).

Aneurysm rebleeding is the most threatening early neurological complication and usually occurs in the first 24 h after the initial hemorrhage (5, 6). However, delayed cerebral ischemia (DCI) remains the major cause of morbidity and mortality among patients who survive after repair of the ruptured aneurysm (7, 8).

For decades, angiographic cerebral vasospasm (ACV), a common neuroradiological finding after SAH, has been proposed as the primary cause of DCI and DCI-related cerebral infarction (9). In turn, severe angiographic vasospasm can cause DCI and DCI-related cerebral infarction, which made the prevention and treatment of ACV a major target in the management of SAH. Crowley et al. analyzed 381 patients who were part of a randomized clinical trial and all of whom underwent serial CT scanning and angiography (10). Angiographic vasospasm was graded as none/mild (209, 55%), moderate (118, 31%), or severe (54, 14%). Delayed cerebral infarctions occurred in 3% of those with no/mild, 10% with moderate, and 46% with severe vasospasm. The data also show that a minority of patients can develop DCI without ACV (10). Angiographic vasospasm occurs in approximately 70% of patients during the first 2 weeks after SAH, but the incidence of DCI is only around 30% (7). Some patients with severe ACV do not develop ischemia or infarction for the same reasons that a patient with a carotid artery occlusion may not, which includes (a) the recruitment of collateral blood flow through the anterior and posterior communicating arteries (i.e., the primary collateral pathway), or through the ophthalmic artery and leptomeningeal vessels (i.e., the secondary collateral pathway) (11, 12) and (b) the cerebral vasoreactivity status (i.e., intact versus impaired) (13). In addition, patients may develop cerebral infarction in vascular territories that are not affected by ACV (14, 15). Moreover, enteral nimodipine is the only pharmacological treatment shown to improve functional outcome, despite the fact that it does not lead to marked cerebral vasodilation (16, 17).

Also, a meta-analysis that included 14 randomized controlled trials in SAH, involving 4,235 patients, showed that the tested drugs significantly reduced angiographic vasospasm, but clinical outcomes remained unaffected (18).

Because of the failure of several pharmacological trials and the dissociation between vasospasm-related morbidity and functional outcomes, other possible mechanisms, such as early brain injury, cortical spreading ischemia, microthrombosis, cerebral autoregulation impairment, and capillary transit time heterogeneity, are thought to play a role in the pathophysiology of DCI and DCI-related cerebral infarction (1, 3, 19, 20). These additional mechanisms tend to be more difficult to assess clinically and the dissociation mentioned above could also be due to drug toxicity and off-target effects, effects of rescue therapy, inadequate trial sample size and other trial design problems, and testing of only one dose and dose regimen.

Another common biochemical mechanism after brain injury is neuroinflammation, which may also contribute to many of the causes of brain damage after SAH. Subarachnoid blood and subsequent hemoglobin degradation can in turn trigger the inflammatory cascade, which also develops in the brain probably secondary to ischemia, blood–brain barrier breakdown, and such. There is an increasing interest in the understanding of the role of neuroinflammation in the pathophysiology of DCI and other severe complications after SAH.

The main goal of this review article is to summarize the concept of neuroinflammation following SAH, and its potential contribution to ACV, DCI, and systemic complications. We also address the possible benefits of targeting the inflammatory cascade after aneurysmal SAH. Finally, we review studies of drugs directed at neuroinflammation after SAH (**Table 1**).

## SEARCH STRATEGY

A PubMed search was performed from inception to November 2017 for articles published in English by using the terms "Subarachnoid Hemorrhage" [Mesh] AND ("Neuroinflammation" [Title/Abstract] OR "inflammation" [Title/Abstract]). A total of 272 articles were found, including 155 articles on human subjects. The authors' own databases additionally were used as a source for this review article. This review was limited to studies in humans, because the literature on experimental animal models is vast, and it is beyond the scope of this article.

## DCI and Angiographic Vasospasm

The majority of patients (approximately 70%) develop some radiological degree of vasospasm between 3 and 14 days after a single SAH (9). However, only 30% of those will progress to develop DCI and even fewer to delayed cerebral infarction (15, 34). While ACV is an important contributor to DCI, there is evidence that other processes are involved (1, 3, 35–38).

Two major neurological consequences of aneurysmal SAH are currently described, early brain injury and ACV/DCI. Early brain injury refers to the acute effects of aneurysm rupture. Arterialized blood flows into the subarachnoid space, which suddenly increases the intracranial pressure. Consequently, the cerebral perfusion pressure is acutely reduced leading to a reduction in cerebral blood flow that produces global cerebral ischemia. Clinically, the phenomenon is characterized by transient loss of consciousness that can progress to intracranial circulatory arrest in severe cases (39, 40). There also are probably effects from the subarachnoid blood as well as brain injury from other physical and biochemical processes. Ischemia with its associated cell death causes influx of inflammatory cells, activation of microglia and neuroinflammation. Patients who survive the initial hemorrhage are at risk of DCI that is the main determinant of unfavorable outcome after SAH (41). DCI is a clinical syndrome characterized by "the occurrence of focal neurological impairment (such as hemiparesis, aphasia, apraxia, hemianopia, or neglect) and/or a decrease of at least two points on the Glasgow coma scale (either on the total score or on one of its individual components, such as eye, motor on either side, or verbal) (7). This should last for at least 1 h, is not apparent immediately after aneurysm occlusion, and cannot be attributed to other causes by means of clinical assessment, computed tomographic, or magnetic resonance imaging of the brain and appropriate laboratory studies". The pathophysiology of DCI is multifactorial and remains to be completely elucidated, however, it is hypothesized that ACV is one if not the primary contributor, along with cortical spreading ischemia, impaired cerebral autoregulation and microcirculation constriction, microthrombosis, capillary transit time heterogeneity, and neuroinflammation (1, 3, 35–38, 42). The risk of DCI is increased by the amount of subarachnoid blood (i.e., the volume, density, and persistence of blood in the subarachnoid space) (43–45);

**TABLE 1** | Summary of medication with anti-inflammatory activity tested in SAH.

| Drug | Design | Dose | Patients | Outcome | Mechanism of action | Conclusion |
|---|---|---|---|---|---|---|
| Cyclosporin A (21) | Prospective cohort study | Loading dose of 7.5 mg/kg Maintenance: enteral administration every 12 h for two doses, to maintain levels of 50–400 ng/kg | Nine patients with Fisher Grade 3 | GOS at 6 months | Prevent vasospasm, inhibit IL-2 production, and prevent T-cell dysfunction | CycA proved safe to use but failed to prevent the development of cerebral vasospasm or delayed ischemic deficits in patients considered at high risk |
| Cyclosporin A (22) | Randomized clinical trial | Cyclosporine A orally 6–9 mg/kg/day to maintain level of cyclosporine in the blood at 100–400 ng/ml | 25 patients (9 received treatment) | *Neurological state* | Prevent vasospasm, inhibit IL-2 production, and prevent T-cell dysfunction | Patients treated with early clipping (up to 72 h after SAH) plus cyclosporine A had significantly better "neurological outcome" than controls |
| Methylprednisolone (23) | Case-control study | Methylprednisolone started within 3 days following the tapering regimen:<br>– 30 mg/kg q6h × 12, 15 mg/kg q6h × 4, 7.5 mg/kg q6h × 4, 3 mg/kg q6h × 4, and 1.5 BID × 2<br>– 30 mg/kg before aneurysm operation | 42 patients (21 received treatment) | | Corticosteroids have multiple anti-inflammatory actions, mostly on chronic inflammation | |
| Methylprednisolone (24) | Double-blind, placebo-controlled, randomized trial | Methylprednisolone 16 mg/kg IV every day for 3 days (started within 6 h after angiographic diagnosis of aneurysm rupture), or placebo | 95 patients | *Symptomatic vasospasm*[a] mRS in living patients GOS at 1 year after SAH, in all patients | Corticosteroids have multiple anti-inflammatory actions, mostly on chronic inflammation | The treatment did not reduce the incidence of *symptomatic vasospasm* but improved functional outcome |
| Hydrocortisone (25) | Double-blind, placebo-controlled, randomized trial | Hydrocortisone 3 g IV BID, repeated 6 times | 140 patients, 71 patients who received hydrocortisone | Mental, speech, and motor function | Hydrocortisone reduces vascular sensitivity to various vasoconstrictive stimuli. It inhibits phospholipase to reduce production of prostaglandins. It stabilizes the cell membrane and prevents cerebral edema | Patients who received hydrocortisone showed improvement in mental, speech, and motor function |
| Dexamethasone (26) | A propensity score analysis | Dexamethasone 4 mg q6h, then tapering down by 1 mg per dose every 24 h until discontinuation | 309 patients, 101 (33%) received treatment | Unfavorable outcome (mRS > 3) | | Dexamethasone was associated with a significant reduction in mRS >3, but its use had no association with DCI or infection |
| Simvastatin (1, 27) | Meta-analysis | Simvastatin 40 or 80 mg/day up to 21 days | Six randomized clinical trials, including 1,053 patients | Delayed ischemic deficit and delayed cerebral infarction | Neuroprotection independent of cholesterol reduction and exclusively associated with upregulation of endothelial nitric oxide synthase | No effect on delayed ischemic deficit, delayed cerebral infarction, mRS ≤2, vasospasm, ICU stay, hospital stay, and mortality |

(*Continued*)

**TABLE 1** | Continued

| Drug | Design | Dose | Patients | Outcome | Mechanism of action | Conclusion |
|---|---|---|---|---|---|---|
| Acetylsalicylic acid (aspirin), ADP $P2Y_{12}$ receptor antagonists (thienopyridines), and thromboxane synthase inhibitors (28) | Meta-analysis | Multiple regimens[b] | Seven randomized clinical trials, including 1,385 patients | Poor outcome (death, or dependence on help for activities of daily living) | Aspirin exerts its antiplatelet activity by the irreversible inhibition of COX-1 enzyme, thereby blocking the formation of thromboxane A2 in the platelets Because aspirin block the COX-1 enzyme, which decreases prostaglandin synthesis, leading to an anti-inflammatory effect Thienopyridines are ADP $P2Y_{12}$ receptor antagonists (e.g., ticlopidine) that inhibit the intracellular pathways leading to platelet activation | No effect on case fatality, aneurysmal rebleeding, poor outcome, secondary brain ischemia, and intracranial hemorrhagic complications. Ticlopidine was the sole antiplatelet agents associated with a significant reduction in the occurrences of a poor outcome (RR 0.37, 95% CI 95% CI 0.14–0.98), however, this result was based on one small RCT |
| Non-steroidal anti-inflammatory (29) | A propensity score-matched study | Multiple regimens not described[c] | 178 patients were matched [89 received non-steroidal anti-inflammatory drug (NSAIDs), 89 did not] | Clinical outcomes included 6-week mortality, 12-week modified Rankin scale (mRS) score, DCI, and delayed ischemic neurological deficit (DIND) | NSAIDs inhibit COX, which decreases prostaglandin synthesis; ibuprofen inhibits expression of endothelial adhesion molecules and reduces subarachnoid inflammation | No significant difference in functional outcome, in the development of DINDs, angiographic vasospasm, or need for rescue therapy |
| Clazosentan (19) | Meta-analysis | Multiple regimens[d] | Four randomized clinical trials, including a total of 2,181 patients | Glasgow Outcome Scale—extended and mortality | Synthetic endothelin A receptor antagonist, with reduction of angiographic vasospasm | Clazosentan had a significant impact in the reduction of DINDs and delayed cerebral infarction. However, functional outcomes or mortality were unaffected Side effects, such as hypotension, anemia, and pulmonary complications may have reduced the beneficial effects of the drug |
| Cilostazol (30) | Randomized, single-blind study | | 109 patients undergoing clipping of ruptured aneurysms | | Selective phosphodiesterase III inhibitor, which inhibits platelets through an increase in intraplatelet cAMP levels. It has an antithrombotic, vasodilatory, anti-smooth muscle proliferation, and cardiac inotropic and chronotropic effects. Cilostazol also exhibits anti-inflammatory properties including inhibiting microglial activation | A multicenter randomized clinical trial of cilostazol has shown a decrease in angiographic vasospasm but no improvement in outcomes 6 months after SAH. Cilostazol significantly reduced angiographic vasospasm, DCI and cerebral infarction but had no effect on outcome |

(*Continued*)

**TABLE 1** | Continued

| Drug | Design | Dose | Patients | Outcome | Mechanism of action | Conclusion |
|---|---|---|---|---|---|---|
| Interleukin-1 receptor antagonist (IL-1Ra) (31) | A small Phase II, double-blind, randomized controlled study | IL-1Ra (500 mg bolus, then a 10 mg/kg/h infusion for 24 h) | 13 patients, 6 patients received IL-1Ra | Primary outcome: change in CSF IL-6 between 6 and 24 h | IL-1Ra limits brain injury in experimental stroke and reduces plasma inflammatory mediators associated with poor outcome | IL-1Ra appears safe in SAH patients. The concentration of IL-6 was lowered to the degree expected, in both CSF and plasma for patients treated with IL-1Ra. This did not reach statistical significance |
| Dual antiplatelet therapy (aspirin + clopidogrel) (32) | Single center retrospective study | Not described | 161 patients (85 patients received) | Frequency of symptomatic clinical vasospasm and DCI and of hemorrhagic complications | Aspirin has an anti-inflammatory and antiplatelet effect thorough the blockade of COX-1 enzyme Clopidogrel is an antiplatelet agent (ADP $P2Y_{12}$ receptor antagonists) | The use of DAPT was associated with a lower risk of clinical vasospasm and DCI in patients treated for SAH, without an increased risk of hemorrhagic complications |
| Albumin (33) | Open-label, dose-escalation, Phase I pilot study | Tier 1 = 0.625 g/kg<br>Tier 2 = 1.25 g/kg<br>Tier 3 = 1.875 g/kg<br>Tier 4 = 2.5 g/kg<br>The treatment was used for 7 days | 47 patients received treatment 20 in Tier 1, 20 in Tier 2, and 7 in Tier 3 | This was a dose-escalation study; therefore, the maximum tolerated dose of albumin was established. Tolerability was based on the rate of severe-to-life-threatening heart failure and anaphylactic reaction. Also, functional outcome at 3 months was assessed | Antioxidant and scavenger properties<br>Modulate apoptosis Enhance microcirculatory blood flow<br>Increase organ blood flow<br>Decrease leukocyte rolling and adherence, and reduce the inflammatory response | Doses up to 1.25 g/kg/day × 7 days were well tolerated. Functional outcome trended toward better responses in those subjects enrolled in Tier 2 compared with Tier 1 (OR, 3.0513; CI, 0.6586–14.1367) |

[a]*Symptomatic vasospasm (DINDs associated with angiographic arterial narrowing or accelerated flow on TCD, or both).*

[b]*Suppositories of 100 mg ASA for 21 days after surgery; ASA 300 mg twice daily orally or rectal retention enema, starting 72 h after admission, before surgery; ticlopidine 100 mg 3 times a day orally for 2 weeks after the hemorrhage; dipyridamole 100 mg/day orally or 10 mg/day intravenously starting immediately after admission; first group 80 mg OKY-046 per day, second group 400 mg OKY-046 per day, by continuous infusion until 10–14 days, starting immediately after surgery; cataclot 1 μg/kg/min via continuous infusion, starting after surgery continued for 8–14 days; Suppositories with 100 mg ASA once daily, starting after surgery for 14 days.*

[c]*Salicylates (aspirin), propionic acid derivatives (ibuprofen, naproxen), acetic acid derivatives (indomethacin, ketorolac, diclofenac), enolic acid derivatives (meloxicam), and selective cyclooxygenase-2 inhibitors (-coxib's).*

[d]*GOS, Glasgow outcome score; mRS, modified Rankin scale; DCI, delayed cerebral ischemia; COX, cyclooxygenase; SAH, subarachnoid hemorrhage; CSF, cerebral spinal fluid.*

by the initial level of consciousness (i.e., early brain injury) (46, 47); and by factors that alter the relationship between the brain oxygen/glucose supply and demand (1).

## Inflammation as an Additional Contributor to DCI

There is recent evidence suggesting that neuroinflammation may play an important role in the damage of cerebral cells after SAH (42, 48). The products of erythrocyte degradation in the subarachnoid space lead to the accumulation of hemoglobin and its products (i.e., methemoglobin, heme, and hemin), which activate toll-like receptor 4, initiating the inflammatory cascade. Microglia, the resident inflammatory cells in the central nervous system, are activated, which triggers the upregulation of a large number of endothelial adhesion molecules, allowing the inflammatory cells to reach the subarachnoid space (49). Macrophages and neutrophils, once in the subarachnoid space, start the process of phagocytosis of the product of degraded red cells, with the attempt of removing the extravascular hemoglobin, the process of which may promote neuronal healing (50). The clearance of hemoglobin from subarachnoid space depends on and is accelerated by the ligation of hemoglobin to haptoglobin. The complex hemoglobin/haptoglobin is than phagocyted by immune cells (51).

Macrophages and neutrophils are indispensable for the clearance of subarachnoid blood, however, they may become imprisoned within the subarachnoid space after the eventual reestablishment of the blood–brain barrier, and also because of the changes occurring in the cerebral spinal fluid (CSF) flow. The main problem with the entrapment of inflammatory cell in the subarachnoid space is that they suffer degranulation, releasing several inflammatory and vasoactive factors, such as endothelins. Also, these inflammatory and vasoactive factors are not restricted to the subarachnoid space, but generalized throughout the central nervous system, inducing cerebritis, aseptic meningitis, and cerebral vasoconstriction (50).

## Monitoring Inflammation After SAH

Several methods exist to monitor systemic and neurological inflammation in the patients with SAH. They include classic systemic signs of inflammation such as fever, leukocytosis, and systemic inflammatory response (SIRS), as well as the evaluation of pro- and anti-inflammatory cytokines in the peripheral blood, CSF, and cerebral extracellular fluid (52).

Increased levels of inflammatory cytokines are found in the CSF, cerebral extracellular fluid, and blood of patients with SAH (53). Two sets of authors reviewed published data reporting biomarkers for assessing outcome after SAH (54, 55). Neuron and astrocyte proteins, cell adhesion proteins, and extracellular matrix molecules, vascular, coagulation, and cardiac proteins have all been measured in various studies of CSF and blood after SAH. Inflammatory substances in blood that have been shown to be elevated after SAH included C-reactive protein (CRP), tumor necrosis factor α (TNF-α), and interleukin-1 receptor antagonist (IL-1Ra) and IL-6 (IL-6). Elevated levels of TNF-α, IL-1Ra, IL-6, and IL-8 were also described in the CSF (56–58). IL-1Ra concentrations were measured in blood and were significantly higher after SAH than in controls, in poor-grade SAH patients compared to good grades and in patients who had unfavorable as compared to favorable outcome (56). DCI further increased the concentration of IL-1Ra.

In one study, CSF TNF-α concentrations increased between 4 and 10 days after SAH in patients with unfavorable outcome whereas they did not if there was favorable outcome (56). The same rough correlation of increased CSF TNF-α and unfavorable outcome was reported by other investigators (59). Similarly, CSF levels of IL-6 and IL-8 were shown to be significantly elevated in SAH patients compared to controls, and this elevation was associated with the development of symptomatic vasospasm between day 5 and 7 (58).

Subarachnoid increases in IL-1, IL-6, and TNF-α may precede the hemodynamic abnormalities detected by transcranial Doppler and to be associated with the development of severe ACV diagnosed by transcranial Doppler ultrasound.

Elevated IL-6 levels also may be an early marker of DCI and unfavorable outcome. Muroi et al. showed in a cohort of 138 SAH patients that early elevation of IL-6 (days 3–7) was significantly associated with the occurrence of DCI and unfavorable outcome, after the adjustment for confounding factors such as infection and therapeutic hypothermia (60). In addition, elevated IL-6 levels detected in the cerebral extracellular fluid by cerebral microdialysis in patients of all grades predicted the occurrence of DCI in one study (61).

Complement system is another important component of inflammatory response after SAH. Complement activation and the formation of membrane attack complex contribute to the development of cerebral edema and the disruption in the blood–brain barrier. Interestingly, the process of complement activation and membrane attack complex formation is also implicated in the inflammation and degradation of the cerebral aneurysm wall, with consequent aneurysm rupture (62).

The complement activation in association with the formation of membrane attack complex may contribute to the development of angiographic vasospasm (63). In one study investigated the role of sequential measurement of serum complements (i.e., CH50, C3, C4) in the first 3 weeks of hemorrhage. Hunt and Hess grade was associated with C4 levels, and C4 levels were also markedly reduced in patients with severe angiographic vasospasm with neurological deficits (64).

Although, the measurement of cytokines and their receptors in the CSF, cerebral extracellular fluid, and blood seem to be feasible, their use remains experimental. Also, inflammation is the fundamental response of tissues to injury and is generally required for tissue healing. There are situations where inflammation is detrimental. Therefore, it is going to be very complex to determine which aspect of cerebral inflammation is beneficial or harmful (52). More adequate, well controlled and thus basically prospective multicenter studies would be needed before we could draw conclusions on the clinical utility of inflammatory biomarkers (53).

## Inflammation, Brain Injury, and Systemic Complications

There is systemic immune response activation after SAH and it is commonly manifested by high levels of circulating cytokines, such as IL-1, IL-6, and TNF-α (65). These are key mediators of systemic inflammation. Clinically, this process is usually manifested by fever, leukocytosis, tachycardia, and tachypnea. These are components of SIRS, which has been defined by the presence of two or more of the following: temperature <36 or >38°C, heart rate >90 bpm, respiratory rate of >20 breaths/min, and white blood cell count of <4,000 or >12,000/mm$^3$ (66, 67). In 2 reports that included 276 and 413 patients with SAH, respectively, SIRS was present on admission in over half and in 63–85% within 4 days (68–70). Its occurrence was associated with poorer neurological grade, larger amount of subarachnoid blood on computed tomography. In one of the two studies, it was an independent predictor of angiographic vasospasm, systemic complications, unfavorable outcome, and death.

Systemic inflammatory response is a relatively nonspecific clinical syndrome that reflects a complex interaction among inflammation, coagulation, sympathoadrenal activation, and endothelial cell activation and dysfunction (71). This complex process generates and perpetuates tissue hypoperfusion, ultimately culminating with microthrombosis and compromised microcirculation blood flow, which is manifested by the multiorgan dysfunction syndrome (71, 72). In addition, catecholamines released into the systemic circulation after SAH may induce cardiac and pulmonary dysfunction (i.e., myocardial stunning and neurogenic pulmonary edema) (73, 74). Sympathoadrenal activation, represented by high circulating catecholamine levels, has also been shown to be highly associated with coagulopathy, endotheliopathy, and functional outcome in patients with isolated traumatic brain injury (75, 76).

Other indirect surrogate markers of systemic inflammatory activity after SAH include hyperthermia, elevated white blood cell count, hyperglycemia, high erythrocyte sedimentation rate, high CRP, transthyretin, and negative nitrogen balance (60, 64, 66, 72–82). Those systemic inflammatory parameters are associated with angiographic vasospasm, DCI, and unfavorable outcome (66, 68).

## Platelet Activation and Inflammation After SAH

Inflammation is intimately related to platelet adhesion, activation of coagulation cascade, and consequently the formation of microthrombi (82). Injury to a blood vessel leads to endothelial platelet adhesion, which triggers the coagulation cascade. Thrombin, von Willebrand factor, and collagen are capable of activating the platelets, which release a large number of procoagulant factors, responsible for the development of a hemostatic clot (82–84).

In SAH patients, microthrombosis was described in an autopsy study of seven patients who died within 3 days of SAH (85). The density of thrombi (microclot burden) was associated with clinical and radiological evidence of delayed ischemia. Also, microclot burden was significantly associated with histological evidence of ischemia.

Additional evidence for microthromboembolism includes a prospective study of SAH patients monitored with transcranial Doppler (86). Microembolic signals were detected in 16 of 23 patients (70%), and 44 of 138 arteries (32%) monitored. Microembolic signals were more common in patients with compared with patients without clinical vasospasm (83 versus 54%). These data at least show an association between inflammation, activation of coagulation cascade and formation of microthrombi after SAH and may be an additional component of DCI (83).

Inflammation and platelet activation have been associated with both early brain injury and DCI after SAH (82, 84). Frontera et al. (82), in a prospective study enrolled 106 consecutive SAH patients, comparing them with 26 control subjects. Compared to controls, SAH patients demonstrated higher levels o inflammatory biomarkers (i.e. higher levels of C-reactive protein), and higher levels of platelets activation by thromboelastography. Platelet activation and inflammation were related to the severity of early brain injury, and it was also associated with the development of DCI and unfavorable functional outcome (82).

Interestingly, nimodipine has been shown to increase endogenous fibrinolysis, which may partially explain the benefit of nimodipine use by the reduction in microthrombosis after SAH (87). However, the exact mechanism by which nimodipine exerts its beneficial effects remains to be determined, but its probably multifactorial, including fibrinolysis, inhibition of cortical spreading ischemia, and the blockage of calcium influx to neurons after ischemia (1, 20).

## Antiplatelet Drugs After SAH

Cyclooxygenase (COX) enzymes are fundamental for the balance of vascular homeostasis (88). Thromboxane A2 ($TxA_2$) is released by platelets as a result of COX-1 activity, and prostacyclin is synthetized mainly in vascular endothelium by COX-2 (**Figure 1**). $TxA_2$ induces platelet aggregation, vasoconstriction, and smooth muscle proliferation, while prostacyclin antagonizes $TxA_2$ effects in the macrovascular endothelium through smooth muscle relaxation and vasodilator effects. Prostacyclin also inhibits platelet aggregation through prostacyclin receptors.

Acetylsalicylic acid [aspirin, a non-steroidal anti-inflammatory drug (NSAID)] inhibits cyclic prostanoid synthesis ($TxA_2$, prostacyclin and other prostaglandins, **Figure 2**). The mechanism of its antithrombotic effect is by irreversible acetylation of COX-1, an enzyme constitutively expressed platelets and most other cells. This inhibits platelet aggregation. Its anti-inflammatory effects are due to inhibition of COX-2, which is induced by inflammation and generates inflammatory prostaglandins.

Dorhout Mees et al. conducted a meta-analysis of seven randomized clinical trials (1,385 patients) that evaluated the effect of antiplatelet drugs on outcome, case fatality, secondary ischemia, hemorrhagic intracranial complications, and aneurysm rebleeding in patients with SAH (28). The studies tested different drugs and regimens, including suppositories or oral aspirin, oral ticlopidine, oral or intravenous dipyridamole, continuous infusion of OKY-046, and continuous infusion of cataclot (90–94). There was no effect of the antiplatelet drugs on any of the outcome measures. Ticlopidine was associated with a significant reduction

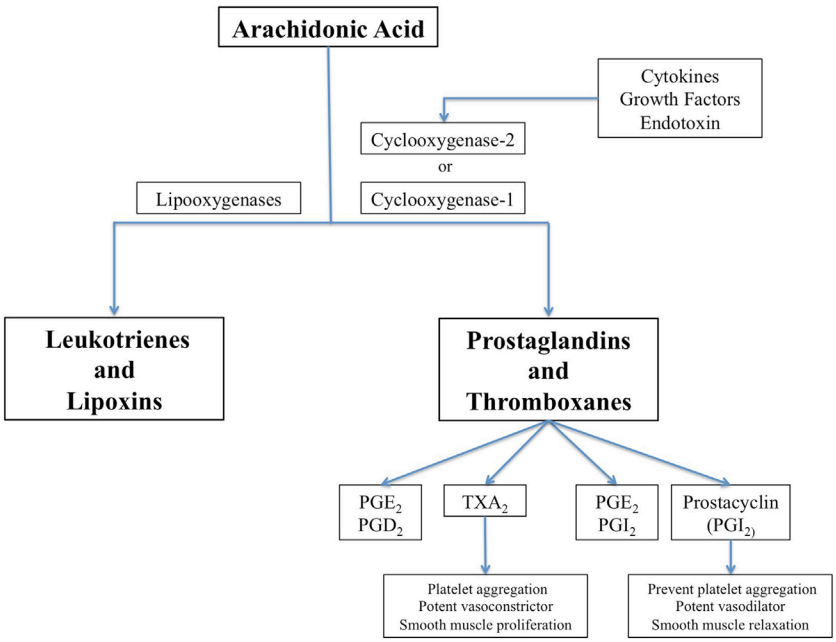


**FIGURE 1** | The biochemical pathway for synthesis of prostaglandins, thromboxanes, leucotrienes, and lipoxins, showing cyclooxygenase (COX)-1 which is constitutively expressed in many cells and COX-2 that is induced by inflammatory stimuli.

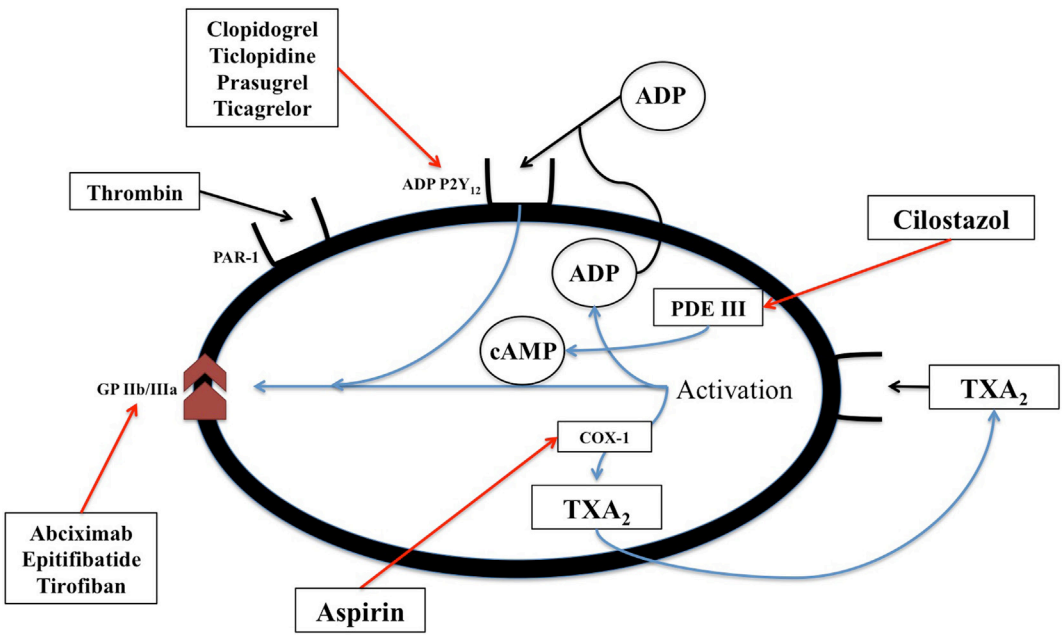


**FIGURE 2** | The biochemical pathways and receptors on platelets along with the site of action of non-steroidal anti-inflammatory drugs as well as some physiologic mediators of platelet degranulation and aggregation. This figure was modified from Angiolillo (89).

in poor outcome but this was based on one randomized trial of 135 patients (95).

## Steroids for SAH

Corticosteroids are anti-inflammatory. They inhibit the formation of adhesion molecules, arachidonic acid metabolites, cytokines, and chemokines. The mechanism of action that accounts for their anti-inflammatory effects is direct binding of the glucocorticoid to the glucocorticoid receptor, forming a glucocorticoid/glucocorticoid receptor complex. This is transported in to the nucleus where it binds to glucocorticoid responsive elements in the promoter region of genes, or interacts with

other transcription factors, in particular activating protein-1 or nuclear factor-κB (96).

Several different types doses and dose regimens of corticosteroids have been studied in patients with SAH. Overall, the studies show mixed results and sample sizes are too small to draw conclusions among other shortcomings of the studies (23–25). The largest study was a retrospective cohort that included 309 patients, 101 (33%) who received dexamethasone. Dexamethasone significantly reduced in the odds of unfavorable outcome although it had no effect on DCI or the rate of infections (26). Glucocorticoids are not recommended for routine use in patients with SAH.

Important to mention is that a large randomized clinical trial, including more than 10,000 patients with moderate-to-severe traumatic brain injury, compared the effect of 48 h infusion of methylprednisolone with placebo. The risk of death from all causes was increased in the intervention group (97, 98). The exact cause of worse outcome with the use of steroids after traumatic brain injury is not well established.

## Immune-Suppressive Treatment in SAH

There are at least two studies that tested the use of immune-suppressive treatment in SAH. The first study was a randomized clinical trial, which included 25 patients (22). Nine received the intervention (cyclosporine A orally 6–9 mg/kg/day to maintain level of cyclosporine in the blood at 100–400 ng/ml). Patients treated with early clipping (up to 72 h after SAH) plus cyclosporine A had significantly better neurological outcome. The second study treated nine SAH patients with Fisher Grade 3 SAH with cyclosporine A (21). Despite the small number of patients, cyclosporine A was considered to be safe but to not prevent ACV or DCI.

Many SAH patients admitted to the critical care unit are exposed to invasive procedures and catheter placement, such as mechanical ventilation, external ventricular drain, invasive intracranial monitors, and vascular catheters. These invasive tools are fundamental for the management of poor-grade SAH patients (1), however, they may increase the risk of hospital-acquired infections (99). Therefore, the use of immunosuppressive treatment, including the use of glucocorticoids, might increase the risk of infection in this patient population, which increases the hospital morbidity and mortality.

## NSAIDs for SAH

Non-steroidal anti-inflammatory drugs inhibit COX (88). Traditionally they inhibited COX-1 and COX-2 although there are some specific COX-2 inhibitors. Thus, they inhibit inflammation mediated by COX-2. While aspirin is the classic NSAID, the effects differ between the newer NSAIDs and from aspirin probably due to the relative potencies against COX-1 and COX-2 and their effects on other targets. For example, ibuprofen reduces the expression of adhesion molecules by the endothelium, which in turn decreases the inflammation in the subarachnoid space. In a propensity score-matched study, Nassiri et al. studied 178 patients with SAH who were matched by propensity scoring into 89 who received NSAIDS and 89 who did not (29). Use of NSAIDs was associated with lower in-hospital mortality and shorter intensive care and hospital stay. There was, however, no significant difference in functional outcome, in the development of delayed ischemic neurological deficits, angiographic vasospasm or need for rescue therapy. Muroi and colleagues found that NSAID use was associated with lower systemic IL-6 and CRP concentrations and with better outcome in 138 patients with SAH (100). There is at least one randomized clinical trial of NSAIDs in 81 patients with SAH (101). Meloxicam had no significant effect on ACV or clinical outcome compared to placebo. Currently, the treatment of SAH patients with NSAIDs cannot be recommended.

## Other Potentially Anti-Inflammatory Drugs

Several other medications with anti-inflammatory activity have been tested in patients with SAH (**Table 1**). The statins are the best studied and have been completely ineffective at least in the doses and dose regimens studied (102). Albumin has multiple systemic and cerebral effects, including antioxidant and scavenger properties; the capacity to modulate cellular apoptosis; enhance of microcirculatory and organ blood flow; and an anti-inflammatory effect through the decrease in leukocyte rolling and adherence. Albumin has been studied in a dose-escalation, Phase I, pilot study (33). Doses up to 1.25 g/kg/day × 7 days were well tolerated, with a trend toward better 3-month functional outcome. A Phase III placebo control trial will be carried out in the near future.

# CONCLUSION

There is an increasing interest in the understanding of the role of neuroinflammation in the pathophysiology of early brain injury and DCI after SAH. Inflammatory biomarkers are associated with the occurrence of ACV, DCI, and unfavorable outcome. However, the use of anti-inflammatory agents was studied in only a small numbers of subjects. Acetylsalicylic acid, other NSAIDs, thromboxane synthase inhibitors, steroids, nitric oxide donors, and immunosuppressant therapies have not shown beneficial clinical effects. On the other hand, none have been studied in enough detail or in adequate, well-controlled clinical trials to reach a definitive conclusion about safety and efficacy. Currently, there is no intervention directly developed and approved to target neuroinflammation after SAH, therefore anti-inflammatory treatments are not suggested in SAH, at least in the doses and dose regimens studied.

# AUTHOR CONTRIBUTIONS

AM wrote the first version of the manuscript. RM made crucial extensive revisions.

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