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Changes in the Lectin Pathway Following Intracerebral or Spontaneous Subarachnoid Hemorrhage

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Abstract

Previous research indicates that the complement system is activated after occurrence of intracerebral hemorrhage (ICH) and spontaneous subarachnoid hemorrhage (SAH). The role of the lectin pathway (LP) of the complement system in this activation has only scarcely been investigated. The aim of this study was to determine the plasma concentration of the LP proteins in patients with ICH or SAH at admission compared to healthy individuals. Secondly, ICH and SAH patients were followed during the initial 24 h of disease, to investigate changes in LP protein concentrations during the critical acute phase. This prospective, observational study included 30 ICH and 33 SAH patients. EDTA plasma samples were collected at admission, 6 and 24 h after symptom onset. Time-resolved immuno-flourometric assays (TRIFMA) were used to measure all proteins of the LP in patient samples and in samples from age- and gender-matched healthy individuals. Compared to healthy individuals, ICH and SAH patients had increased levels of H-ficolin ($p = 0.04$, $p = 0.03$), M-ficolin (both $p < 0.0001$), and MAp44 (both $p = 0.01$) at admission. M-ficolin, H-ficolin, CL-L1, MASP-1, MASP-3, and MAp44 decreased significantly in both ICH and SAH patients during the initial 24 h after symptom onset. In conclusion, we observed significant differences in lectin pathway protein concentrations between patients with ICH or SAH and healthy individuals. Significant dynamics in lectin pathway protein levels were demonstrated during the initial 24 h after symptom onset. This indicates a potential role of the LP proteins during the acute phase of SAH and ICH.

Keywords Aneurysmal subarachnoid hemorrhage · Complement system · Intracerebral hemorrhage · Lectin pathway

Introduction

Worldwide, stroke is a growing and leading cause of morbidity and mortality [1]. As much as 15–30% of all strokes are caused by spontaneous intracerebral hemorrhage (ICH) or subarachnoid hemorrhage (SAH) [2–4]. The incidence of ICH is 10–30 per 100.000 per year [5, 6] and SAH incidence account for 9–20 per 100.000 per year [2]. Current treatment options are limited and a poor prognosis is the reality for both patient groups. With a 30-day mortality between 25 and 48%, new therapeutic targets are warranted [1].

ICH occurs from rupture of small penetrating arteries that originates from basilar cerebral arteries [4]. SAH originates from ruptured intracranial aneurysms in 99% of cases [7]. Animal studies demonstrate that a hemorrhage in the brain suddenly exposes brain tissue to elements of the blood including coagulation factors and proteins of the immune system [8]. The coagulation cascade is activated and the blood-brain barrier is disrupted [8–10]. Apoptosis and inflammatory processes are triggered, which leads to edema after the initial hemorrhagic injury [11]. These events are subsequently followed by activation of several blood-borne cascades including activation of the complement system [12, 13].

There are three pathways leading to complement activation: the classical pathway, the alternative pathway, and the

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lectin pathway (LP) [14]. Each pathway is distinct in terms of initiation and leads to a common pathway. The LP is activated when one of the pattern recognition molecules (mannose-binding lectin (MBL), ficolins or collectin liver 1 (CL-L1)) recognizes carbohydrates on the surface of invading pathogens or exposed on damaged or apoptotic cells [15]. Activation of the LP and the complement system evokes an inflammatory response [15] and plays a detrimental role in different kinds of diseases [15–17]. The balance between activation and inhibition of the complement system is critical in controlling the inflammatory response. Recently, it was demonstrated that when the LP is inhibited in experimental ischemic and hemorrhagic stroke, functional outcome was improved [18–20]. While increasing evidence exists supporting the role of the LP in the pathophysiology of ischemic stroke [12, 21], inadequate data is accessible regarding the influence of complement-mediated inflammation after SAH and ICH.

Most of the current knowledge about the complement system following ICH is based on experimental animal studies in mice [19, 22] and rats [20, 23]. Only one study has investigated complement factor C3 in humans after spontaneous ICH [24], and it primarily focused on the risk of rebleeding. No significant difference was found between ICH groups with or without rebleeding regarding C3 [24]. Complement C3-sufficient mice exhibited reduced brain edema compared to complement-deficient mice [22]. Injection of a C5a receptor antagonist showed neuroprotective effects in mice with experimental ICH [19], and reduced brain edema 24 h after intracerebral injection of a complement inhibitor has been observed [23]. Complete complement depletion by cobra venom factor attenuated brain edema after experimental ICH in rats [20]. To our knowledge, no literature exists on the lectin pathway and patients with ICH.

Complement activation after SAH has been investigated in both experimental models and in humans [25–31]. The studies have demonstrated activation of the complement system [25], increased deposition of membrane attack complexes in ruptured aneurisms [26], and elevated C3a, C4a, and C5a after SAH in plasma and cerebrospinal fluid (CSF) [27, 28]. MBL levels were observed to be higher in SAH patients compared to healthy volunteers and associated with non-survival [29]. Zanier et al. assessed the involvement of the ficolins and MBL after SAH [30]. They reported no difference in protein concentrations between patients with SAH and healthy controls. However, low H-ficolin concentrations correlated to the severity of SAH, clinical vasospasms or ischemic lesions, and unfavorable outcome after 6 months. A recently published study reported a significant reduced concentration of MAp44 in SAH compared to controls measured 24 h after symptom onset of SAH in plasma and cerebrospinal fluid [31]. In the same study, they observed increased M-ficolin levels associated with initial worse neurological conditions, identification of vasospasms, and acute cerebral ischemic lesions at follow-up.

The aim of the present study was to determine the plasma concentration of the LP proteins in patients with ICH or SAH at admission compared to protein concentrations in healthy individuals. Secondly, we aimed to investigate changes in protein concentrations during the acute phase of ICH and SAH in the initial 24 h after admission. Finally, we aimed to determine whether plasma concentrations of LP proteins at admission were associated with 30-day mortality. We hypothesized that in both ICH and SAH patients, a change in LP protein concentrations would be observed in the acute phase of the disease reflecting activation of the complement system through the LP.

Methods

Study Populations

We performed a prospective cohort study including ICH and SAH patients between June 2014 and August 2015. The patients were enrolled at the Department of Neurology and the Department of Neurosurgery, Aarhus University Hospital, Denmark. Inclusion criteria were diagnosis of either ICH or SAH by computed tomography (CT) or magnetic resonance imaging (MRI), admission < 6 h after symptom onset, age older than 18 years. Patients were excluded from the study if they met any of the following criteria at admission: pregnancy, treatment with antithrombotic drugs including antiplatelet therapy, active cancer or chemotherapy within the past 3 months, known bleeding disorder, hemorrhage triggered by a structural cerebral cause (arterial-venous malformation, brain tumor, or skull trauma), ischemic stroke within the past 3 months, known liver cirrhosis, or current infection indicated by antibiotic treatment.

Blood samples from healthy individuals were collected from The Blood Bank, Aarhus University Hospital during November and December 2014 [32]. Healthy individuals were age and gender matched to the included patients. A written informed consent was obtained from all patients or next of kin.

The present study was approved by the Central Denmark Region Committees on Health Research Ethics (legally competent patients case no.: 42 457 (4_270514) and appendix: 46 215 (2_17022015) and legally incompetent patients case no.: 41 880 (3_05052014) and appendix: 46 213 (2_19022015)) and The Danish Data Protection Agency (legally competent patients case no.: 1–16–02-224-14 and legally incompetent patients case no.: 1-16-02-225-14). The Helsinki Declaration was followed in all aspects of the study.

Blood Sampling

Each blood sample was collected in a 4.0-mL EDTA tube. Samples from ICH patients and SAH patients were collected at admission within 6 h of symptom onset, as well as 6 and

24 h after symptom onset (Table 2). One SAH patient's admission blood sample was obtained 24 h after symptom onset and was therefore recorded as a 24-h sample. Blood samples rested for at least 30 min before they were centrifuged at 3100×*g* for 25 min and then stored at – 80 °C.

Laboratory Analyses

In EDTA plasma, we measured M-ficolin, H-ficolin, MBL, CL-L1, MBL-associated serine protease 1, 2, and 3 (MASP-1, MASP-2, and MASP -3), mannose-binding lectin-associated protein of 44 kDa (MAp44), and mannose-binding lectin-associated protein of 19 kDa (MAp19). All analyses were performed using in-house developed time-resolved immunoflourometric assays (TRIFMA). For each assay, different coating buffers, dilution agents, and incubation conditions were applied as previously described [33–39]. In brief, proteins were quantified by coating and incubating Flouronunc microtitre plates (NUNC®, Denmark) with specific antibody against the desired protein (or mannan in the case of MBL). A blocking step with human serum albumin was hereafter used to reduce unspecific protein binding. Diluted standards, controls, and samples were added in duplicate. The plates were then incubated with biotinylated anti-protein-antibody and later europium-labeled streptavidin. Finally, enhancement buffer was added before the plates could be read using a VICTOR™ X5 reader (PerkinElmer®, Hamburg, Germany). Internal controls were applied to all plates. The inter-assay coefficient of variation did not exceed 15% for any of the assays except M-ficolin (control 1, 25%; control 2, 12%; and control 3, 7%) and H-ficolin (control 1, 5%; control 2, 19%; and control 3, 33%).

Clinical Data

Upon admission, Glasgow Coma Scale (GCS) and either National Institute of Health Stroke Scale (NIHSS) for ICH patients or The Hunt Hess scale for SAH patients were registered for all patients to evaluate disease severity. The follow-up period was 30 days. Functional outcome at 30 days was assessed by Modified Rankin scale and Barthel index. The 30-day mortality was registered based on medical records. Modified Rankin scale was determined at discharge for all patients if discharge occurred before day 30. When possible, follow-up was also done at day 30. Modified Rankin scale ≥ 3 after 30 days was considered unfavorable outcome. In four ICH patients and five SAH patients, the Modified Rankin scale score at discharge was used, as a Modified Rankin scale score was lacking at day 30.

Statistics

The primary outcome was LP proteins in patients with ICH or SAH at admission compared to protein concentrations in healthy individuals. The sample size calculation was based on MASP-2 levels in a population of 300 Danish blood donors [32]. We chose the minimum relevant difference between patients and healthy individuals to be 140 ng/ml. With a significance level at 5% (2α) and a test power at 90% (1-β), a minimum of 49 patients should be included.

The mixed model of repeated measurements was used to estimate differences in plasma concentration between groups and changes over time. Model validation gave no reason to reject the model, when Gaussian distribution was approximated by logarithmically transforming plasma concentrations of H-ficolin, M-ficolin, CL-L1, MASP-1, MASP-2, and MASP-3. MBL, however, was an exception. Because of a high frequency of MBL-deficiency, causing extremely skewed data, we had to add a variance function to the above model that exponentially depended on the fitted values (this was done using R). Post hoc tests were performed to estimate differences between ICH, SAH, and healthy individuals and the change over time.

Comparison of protein levels between survivors and non-survivors and comparison of protein levels and 30-day mortality were analyzed by a Mann-Whitney test. Analyses were performed in R® (version 3.2.2 2015) and Stata14® (StataCorp, Texas, USA). Graphs were drawn using GraphPad Prism® version 6 (Graph Pad Software, California, USA). All p values < 0.05 were considered statistically significant.

Results

Clinical Characteristics and Patient Inclusion

In total, 63 patients were included in the study. Demographics and clinical characteristics are presented in Table 1. The main part of patients included was women (ICH 70%, SAH 73%). Median age in ICH patients was 64 and 58 years in SAH patients.

The prior diagnosis of hypertension was 50% in ICH patients compared to 15% of all SAH patients. At admission, ICH patients' median GCS score was 13 with a wide range and the median NIHSS score was 17. The median GCS score was 13 in SAH patients, also with a wide range, and the median Hunt Hess scale was 3. Only a minor part of ICH patients underwent invasive procedures compared to SAH patients, and 88% of SAH patients were treated with tranexamic acid (TXA) within the first 24 h after symptom onset. Patients received between 0 and 6 doses of 1 g TXA during the first 24 h after symptom debut (Supplementary Fig. 1). Thirty-day mortality was higher for SAH patients (30%) compared to ICH patients (20%). Standard laboratory analysis all fell within the reference interval for both SAH and ICH patients.

Table 1 Demographics and characteristics of intracerebral hemorrhage and spontaneous subarachnoid hemorrhage study cohorts. Data are expressed as median (interquartile range) or numbers (%)

Variables	ICH ($n = 30$)	SAH ($n = 33$)
Demographics		
Age, years	64 (54–75)	58 (52–68)
Gender (n = women)	21 (70%)	24 (73%)
Smoking ($n = 22_{ICH}/20_{SAH}$)	6 (27%)	9 (45%)
Alcohol consumption[a] ($n = 25_{ICH}/26_{SAH}$)	3 (12%)	1 (4%)
Hypertension (n)	15 (50%)	5 (15%)
Clinical characteristics at admission		
Blood pressure systolic (mmHg)	167 (145–182)	150 (137–177)
Mean arterial pressure (mmHg)	113 (99–128)	105 (93–119)
Pulse (per minute)	79 (72–92)	73 (61–86)
Cerebral pressure gauge	2 (7%)	3 (9%)
Glasgow Coma scale (3–15)	13 (10–15)	13 (5–15)
NIHSS (0–42)	17 (7–21)	–
Hunt Hess scale (1–5)	–	3 (1–4)
Laboratory variables at admission (reference interval)		
Hemoglobin (mmol/L) (7.3–10.5)	8.7 (8.0–9.4)	8.3 (7.8–8.6)
Platelet count ($\times 10^9$/L)[b] (135–400)	244 (201–270)	217 (173–268)
eGFR (mL/min) (> 60)	≥ 90 (89–90)	89 (79–90)
Alanine transaminase[b] (U/L) (10–70)	19 (14–24)	22 (18–29)
C-reactive protein (mg/L) (< 8)	3 (1–7)	2 (1–4)
Treatment within 24 h		
Tranexamic acid	0	29 (88%)
External ventricular drain	4 (13%)	10 (30%)
Coil	0	12 (36%)
Clips	0	13 (39%)
Evacuation hematoma	2 (7%)	2 (6%)
Decompressive craniotomy	0	5 (15%)
30-day outcome		
Modified Rankin score at discharge (1–6)	4 (3–5)	4 (1–6)
Modified Rankin score day 30 (1–6)	4 (3–5)	5 (2–6)
Barthels index day 30 (0–100)	63 (31–94)	80 (19–100)
30-day mortality (n (%) = non-survivors)	6 (20%)	10 (30%)

ICH, intracerebral hemorrhage; *SAH*, subarachnoid hemorrhage; *eGFR*, estimated glomerular filtration rate; *NIHSS*, National Institutes of Health Stroke Scale

[a] Daily alcohol intake above recommended consumption

[b] Reference interval in common for men and women

Patients were included as planned (Table 2) and very few patient samples fell out-side the accepted time-intervals for inclusion.

Table 2 Time for blood sampling in patients with intracerebral hemorrhage (ICH) or subarachnoid hemorrhage (SAH). Data are presented in median hours and minutes with (interquartile range)

	ICH ($n = 30$)	SAH ($n = 33$)
Admission sample (h min)	3.35 (2.42–4.22)	3.50 (2.38–6.31)
Sample no. 2 (h min)	6.40 (5.44–7.34)	6.23 (5.32–8.29)
Sample no. 3 (h min)	23.27 (22.19–24.12)	23.57 (22.47–24.37)

h, hours; *min*, minutes; *IQR*, interquartile range

Protein Analyses

Concentrations in plasma of M-ficolin, H-ficolin, and MAp44 were significantly higher in both ICH and SAH patients than in healthy individuals (Table 3). MASP-1 concentrations were

Table 3 Complement protein levels in healthy individuals and in patients with intracerebral hemorrhage (ICH) or subarachnoid hemorrhage (SAH). Data are expressed as median and (interquartile range)

Protein, mg/mL	Healthy individuals	ICH at admission	*p* value[b]	SAH at admission	*p* value[b]
MBL	1.13 (0.58–2.44)	1.14 (0.27–2.03)	0.67	1.74 (0.54–3.09)	0.52
M-ficolin[a]	4.14 (3.38–5.10)	6.13 (4.86–12.70)	< 0.001	7.06 (5.18–9.93)	< 0.001
H-ficolin	35.00 (28.89–39.53)	40.38 (35.38–48.44)	0.004	38.40 (34.53–43.31)	0.03
CL-L1[a]	0.53 (0.50–0.59)	0.54 (0.49–0.60)	0.37	0.53 (0.48–0.61)	0.56
MASP-1[a]	7.98 (6.98–9.18)	9.64 (7.86–11.54)	0.02	8.77 (7.05–10.10)	0.38
MASP-2[a]	0.49 (0.39–0.59)	0.58 (0.33–0.78)	0.77	0.53 (0.37–0.71)	0.94
MASP-3[a]	6.55 (5.45–8.05)	5.83 (5.09–6.88)	0.19	6.11 (5.24–7.51)	0.11
MAp44	2.10 (1.89–2.48)	2.47 (2.09–3.12)	0.004	2.35 (1.99–2.98)	0.01
MAp19	0.50 (0.44–0.54)	0.53 (0.42–0.62)	0.10	0.47 (0.37–0.55)	0.06

ICH, intracerebral hemorrhage; *SAH*, subarachnoid hemorrhage; *MBL*, mannan-binding lectin; *CL-L1*, collectin L1; *MASP*, MBL-associated serine protease; *MAp*, mannan-binding lectin-associated protein

[a] Data were logarithmic transformed to fit the mixed model. The *p* value was calculated based on the relatively difference in protein levels

[b] *p* values describe the difference in protein level between patients and healthy individuals

also higher in patients, however, only significant for the SAH group. MASP-3 concentrations were lower in both SAH and ICH patients than in controls although not statistically significant. MBL, CL-L1, MASP-2, and MAp19 displayed no difference in concentration between controls, SAH, and ICH patients.

During the initial 24 h after symptom onset, a significant fall in LP protein concentration was detected for all proteins in the SAH patient group (Fig. 1). In the ICH group, a significant reduction in protein concentration was observed for CL-L1, M-ficolin, H-ficolin, MASP-1, MASP-3, and MAp44. None of the proteins displayed an increase over time. No significant difference or association was observed between number of TXA doses and protein concentrations (Supplementary Fig. 1).

Markers of Inflammation: CRP and M-ficolin

As CRP is a recognized marker of inflammation, LP protein correlation to CRP was assessed for all proteins (Supplementary Fig. 2). The LP protein that displayed the strongest associated with CRP was M-ficolin. Changes in M-ficolin concentration in relation to changes in CRP for ICH and SAH are depicted in Fig. 2a, b. Interestingly, M-ficolin was the only LP protein significantly correlated with the Hunt Hess score for the SAH patients (Fig. 2c).

Protein Concentrations in Survivors and Non-survivors

No consistent link between protein levels at admission and mortality was demonstrated in any of the proteins except MASP-3. MASP-3 showed a higher protein concentration in non-survivors than in survivors at admission ($p = 0.05$) (data not shown).

Discussion

In this study of LP proteins during the initial 24 h after disease onset, we found the concentrations of several LP proteins to be significantly higher in ICH/SAH patients than in healthy individuals. For SAH patients, all LP proteins displayed a significant reduction in concentration during the acute phase of the disease, and M-ficolin concentrations were correlated to CRP levels and also to Hunt Hess score at admission.

The study populations in the present study were representative of cohorts previously described regarding ICH and SAH [4, 40]. We observed that cerebral hemorrhage most often occurs in women above the age of 50 with a median age higher in ICH patients than SAH patients, and with a large number of the ICH patients having a diagnosis of hypertension, consistent with previous studies of ICH and SAH [4, 40]. As expected, non-survivors had significantly lower GCS at admission than survivors. Standard laboratory markers of liver and kidney function all fell within the reference interval for both SAH and ICH patients. Organ affection can thus not explain the changes observed in LP protein concentrations.

The majority of SAH patients received TXA at least once. Activation of the coagulation system has been linked to complement activation [16, 41]. Serine proteases of both the coagulation systems and complement system seem able to activate proteins of the opposite system. This also holds true for plasmin. Activation of plasmin is prevented by TXA [16, 41]. Plasmin has been described as a C5 convertase [42]. Thus, TXA treatment following SAH could potentially influence the lectin pathway proteins and complement activation through the coagulation cascade.

In both SAH and ICH patients, we observed higher levels at admission of M-ficolin, H-ficolin, MASP-1, and MAp44 compared to healthy individuals. Whether this pattern

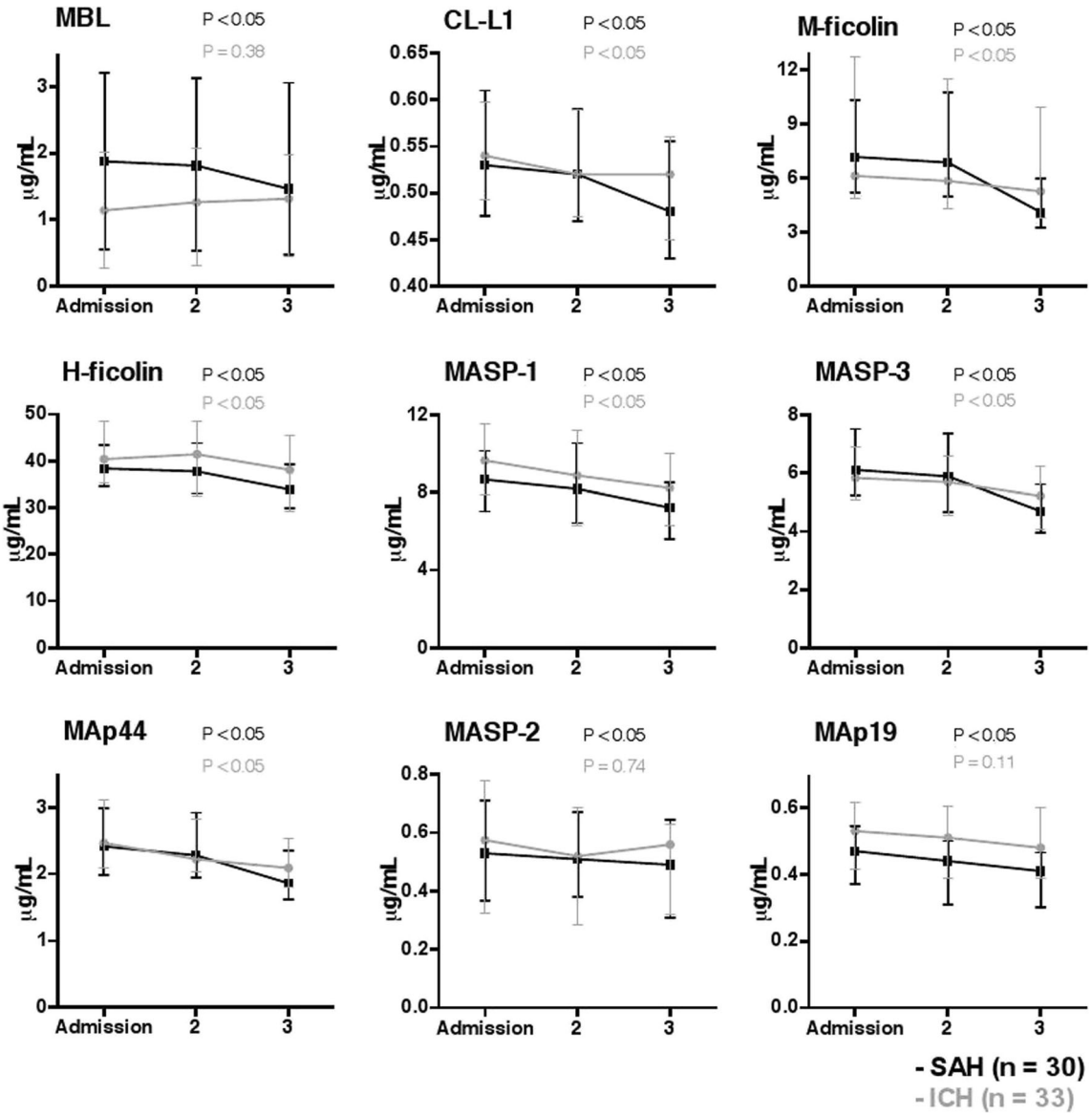

Fig. 1 Changes in lectin pathway protein concentrations during the initial 24 h after intracerebral hemorrhage (ICH) or subarachnoid hemorrhage (SAH)

represents a pattern of people at risk of hemorrhagic stroke or an upregulation of the proteins in the acute phase of the bleeding, we are not able to answer with the present study. Zanier et al. also observed higher concentrations of M-ficolin in SAH patients compared to healthy controls, but did not find a difference in concentrations regarding H-ficolin [30]. However, they showed that low H-ficolin lectin pathway activity was linked to an unfavorable outcome in SAH. H-ficolin is the most abundant of the pattern recognition molecules of the LP [32] and high levels of H-ficolin has previously been described as a disadvantage in conditions where inflammation is ongoing as seen in systemic lupus erythematosus [43, 44] and in conditions of ischemia reperfusion injuries [45]. It is possible that high H-ficolin concentrations enable the potential for excessive complement activation in situations of ongoing inflammation, which would be a disadvantage in ICH and SAH.

Zhang et al. showed that MBL concentrations were higher in acute ischemic stroke patients than in healthy individuals [46], and MBL was suggested as a biomarker to predict outcome following ischemic stroke [46, 47]. We did not observe a difference in MBL concentration between ICH or SAH and healthy individuals, which was also the case in the studies by Llull et al. and Zanier et al. [30, 31].

When comparing our results to the few other studies on the subject, it is important to realize the different study designs [22–31]. This makes the results somewhat challenging to compare, i.e., our admission blood sample was obtained within 6 h after symptom onset compared to 3 days in the study by Zanier et al. [30] and 1 day in the study by Llull et al. [31].

M-ficolin is abundant in peripheral blood monocytes and granulocytes and unlike most other complement proteins is not a liver-derived protein [48]. We found higher

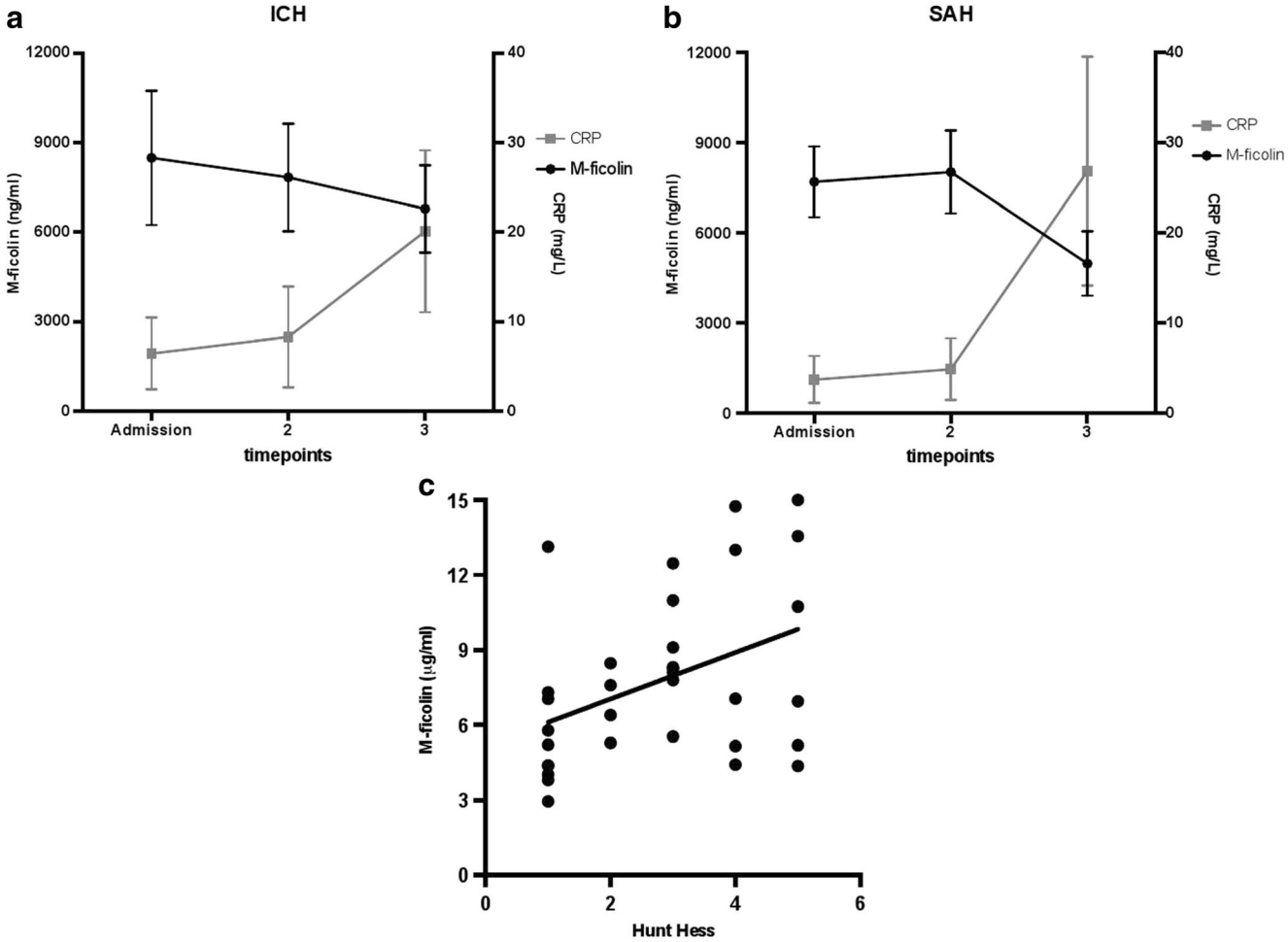

Fig. 2 Markers of inflammation: CRP and M-ficolin. **a** Median change in CRP and M-ficolin during the initial 24 h after ICH. **b** Median change in CRP and M-ficolin during the initial 24 h after subarachnoid hemorrhage (SAH). **c** Correlation between Hunt Hess scale and M-ficolin for patients with SAH at admission. Time points: 1 = at admission, 2 = 6 h after admission, and 3 = 24 h after admission

concentrations in SAH and ICH patients than in healthy individuals as was also the case in the study by Llull et al. [31]. We observed a correlation with the Hunt Hess score at admission (Fig. 2c), and we also observed a negative correlation between M-ficolin and CRP in both SAH and ICH patients (Fig. 2a, b). CRP was increased for all patients during the first 24 h after symptom onset, supporting the hypothesis of increased inflammation after hemorrhagic brain injury.

In SAH patients, all proteins showed a significant reduction in concentration during the initial 24 h (Fig. 1). These results could indicate a consumption of all measured lectin pathway proteins as the inflammatory processes takes off during the acute phase following SAH. Similar results were observed for ICH patients albeit not as pronounced as for the SAH patients. This supports the hypothesis that the LP is activated following SAH and ICH, and also indicates that the inflammatory mechanisms activated during a brain bleed are similar independent of the type of hemorrhage. The observations could support a more equal treatment-approach in the two conditions regarding the prevention of secondary brain injury.

In general, the LP protein concentrations vary extensively between individuals [32], which we also found to be true in our cohorts. Concentration differences between patients and controls and concentration reduction over time, although significant, were relatively small, and the wide range for all the LP proteins makes the use of LP proteins as biomarkers for outcome in SAH and ICH questionable at this point.

We observed an association between high MASP-3 concentrations and mortality following cerebral hemorrhage. Studies have shown that MASP-3 activates pro-factor D to factor D [49–51], thereby directly influencing the alternative pathway. The alternative pathway can function as an amplification loop of the LP pathway. On cells lacking complement inhibitors, the alternative pathway generates 90% of the C3b generated during complement activation [15]. Thus, it is potentially a disadvantage for a person struck by ICH or SAH to a priori have high concentrations of MASP-3 leaving the potential to increase complement activation through the alternative pathway. Although much research is still needed to fully understand the role of MASP-3 in alternative pathway

activation, it represents a potentially interesting target for controlling damage following ICH or SAH.

This study is the first attempt of assessing all LP proteins in SAH and ICH in a human trial during the acute onset of the diseases. The mechanisms that lead to the secondary brain injury of these patients are, at large thought, caused by the inflammatory processes initiated at the sight of injury, which is why the design of this study is one of its major strengths. We analyzed nine LP proteins in the present study, collecting three blood samples during the initial 24 h of SAH and ICH, which enables us to get the full picture of the LP pathway proteins during the initial stages of hemorrhagic brain injury. The laborious and difficult design of the study also had some limitations. First, an unknown number of ICH and SAH patients were missed. This represents a possible selection bias if the patients were not randomly missed. It is possible that the included patients were skewed towards a better prognosis, since patients with cerebral hemorrhage that were found dead or died upon admission were not included. Finally, our follow-up period was limited to 30 days.

In conclusion, a significant difference in protein levels between ICH and SAH patients and healthy individuals were observed regarding M-ficolin, H-ficolin, MASP-1, and MAp44 concentrations. Additionally, significant dynamics in LP protein concentrations were demonstrated during the initial 24 h after symptom onset. This indicates a potential role of the LP proteins in the initial disease processes during the acute phase of SAH and ICH.

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Compliance with Ethical Standards

The present study was approved by the Central Denmark Region Committees on Health Research Ethics (legally competent patients case no.: 42 457 (4_270514) and appendix: 46 215 (2_17022015) and legally incompetent patients case no.: 41 880 (3_05052014) and appendix: 46 213 (2_19022015)) and The Danish Data Protection Agency (legally competent patients case no.: 1–-16–-02-224-14 and legally incompetent patients case no.: 1-16-02-225-14). The Helsinki Declaration was followed in all aspects of the study.

Conflict of Interest None of the authors have any conflicts of interest regarding the present paper but have the following general conflicts of interest: AMH has received speaker's fee from CSL Behring, Bayer, Bristol-Myers Squibb, and Leo Pharma and unrestricted research support from Octapharma, CSL Behring, and Leo Pharma. The remaining authors have no conflict of interest.

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