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ORIGINAL ARTICLE

Activation of Platelets and Occurrence of Cerebral Vasospasm and Delayed Cerebral Ischemia Following Subarachnoid Hemorrhage in a Prospective Pilot-Trial

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SUMMARY

Background: **Aneurysmal subarachnoid hemorrhage (SAH) often leads to poor outcome. The aim of the study was to assess platelet function in patients after SAH.**
Methods: **In this prospective observational study in patients suffering from SAH, platelet count and aggregability were assessed by multiple electrode aggregometry (MEA) over 14 days.**
Results: **In 12 of 18 patients, cerebral vasospasms (CVS) were diagnosed; of those, five developed delayed cerebral ischemia (DCI). We observed a significant increase in the platelet count compared to baseline from day 8 onwards ($p < 0.037$) and, in patients with CVS and DCI, a significant difference in outcome classified by the mRS ($p = 0.047$). Repeated measures ANOVA determined no differences in platelet aggregability in patients with or without CVS/DCI.**
Conclusions: **Besides an increase in platelet count, we detected no increase in platelet aggregability. Nevertheless, patients after SAH may have increased platelet aggregability, which is not reflected by MEA.**
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INTRODUCTION

The development of delayed cerebral ischemia (DCI) is still a major concern in patients suffering from aneurysmal subarachnoid hemorrhage (SAH) [1]. DCI occurs in up to 30% of patients after SAH, representing a significant cause of poor functional outcome [2,3]. Though DCI was initially considered to be the result of cerebral vasospasms as detected by angiography, this paradigm is changing as other mechanisms such as early

brain injury, spreading depolarizations, inflammation, and microvascular disturbances combined with microthrombi have been brought into focus [4,3,5-7].
Both, experimental and human studies revealed an early aggregation of platelets soon after aneurysm rupture [8-10] or described that platelets are activated by endothelial damage in large-artery vasospasm [11]. Further, a systemic procoagulopathic state has been suggested in SAH [12].
The goal of this pilot study was to examine platelet aggregability after aneurysmal SAH in patients with the occurrence of CVS and DCI. Multiple electrode aggregometry (MEA) using the Multiplate® analyzer (Roche AG, Grenzach, Germany) was used as a bedside method for the first time in SAH patients. It analyzes platelet aggregation in response to different agonists at the bedside [13]. MEA was used in various studies to examine the effects of temperature, acidosis, ultrafiltration, colloids, anticoagulants, and antifibrinolytics on platelet aggregation. Additionally, MEA is increasingly used for the perioperative monitoring of platelet function, especially in cardiac surgery [14]. The aim of the present investigation was to study whether there are any changes in platelet function measured by MEA after aneurysmal SAH.

MATERIALS AND METHODS

Trial design
This prospective single-center observational study complies with the Declaration of Helsinki and was approved by the local Scientific and Ethics Review Board (reference number 315/13). To address the function of the platelets, daily blood samples were examined by MEA starting immediately on admission until day 14 after SAH. Platelet counts were documented daily.

Participants
Patients (age 18 years or older) admitted to our center within 6 hours after aneurysm rupture were eligible for this study. Confirmed consent was obtained by the patient or his/her legal representative. Exclusion criteria were (1) admission more than 6 hours after aneurysm rupture and (2) SAH due to other reasons. Demographic and clinical data were recorded. Treatment with antiplatelet medication prior to admission was documented.

Primary endpoint
Primary endpoint was AUC as measured by MEA in three different tests: *in-vitro* aggregability of platelets following stimulation with thrombin receptor activating peptide (TRAP-test), arachidonic acid (ASPI-test), and adenosine-diphosphate (ADP-test). Correlation analysis between platelet function and the occurrence of CVS or DCI was performed. Secondary endpoints were platelet count and outcome as measured by the modified Rankin scale (mRS) assessed six months after SAH.

Multiplate testing/hematological analyses
Blood samples for hematological analyses were drawn using an arterial cannula that was routinely placed in each patient. For MEA analyses, the blood was collected in heparin-anticoagulated and calcium-balanced tubes. For conventional laboratory analyses, sodium citrate-anticoagulated as well as EDTA tubes were used. At each MEA measuring point, blood gas analyses were performed to assess physiologic preconditions for hemostasis (pH, temperature, plasma concentration of ionized calcium, and hemoglobin).
Conventional laboratory coagulation analyses included the assessment of platelet count, hemoglobin concentration, fibrinogen concentration, international normalized ratio (INR), and activated partial thromboplastin time (aPTT). Analyses were performed using fully automated analyzers, STA-R Evolution (Roche AG, Grenzach, Germany) and Sysmex XE 2001 (Sysmex GmbH, Norderstedt, Germany). Standard quality control procedures for each device were routinely performed following the manufacturer's recommendations.

Multiple electrode aggregometry
Methodology of MEA using the Multiplate device has comprehensively been described elsewhere [15,13]. Platelet aggregation was induced following stimulation with 32 mmol/L thrombin receptor activating peptide (TRAP-test), 0.5 mmol/L arachidonic acid (ASPI-test), and 6.4 mmol/L ADP (ADP-test). The extent of platelet-aggregability is expressed by the area under the aggregation curve (AUC), which is presented as arbitrary units called "aggregation units" (U). The reference ranges were defined in accordance to earlier publications [16].

Patient management
After diagnosis of SAH, a digital subtraction four-vessel-angiogram (DSA) was routinely performed. Hemodynamic target values included a cerebral perfusion pressure greater than 60 mmHg and the correction of hyponatremia and hypovolemia. Aneurysms were treated by clipping or coiling at the decision of an interdisciplinary neurovascular team based on the aneurysm's characteristics and the clinical condition of the patient. Acute hydrocephalus was treated by surgical insertion of an external ventricular drainage system. Early aneurysm occlusion (within 24 hours) was strived for in all patients unless in hemodynamically unstable or moribund patients. After securing the aneurysm, all patients were treated in the neurosurgical intensive care unit. All patients received nimodipine from the day of admission either orally (6 x 60 mg/day) or intravenously (2 mg/hour). Screening for CVS included daily clinical examination and transcranial Doppler ultrasound measurements (TCD). If CVS was suspected, the patient received additional imaging such as CT, MRI including angiographic and sometimes perfusion studies or DSA. In case of confirmed CVS, we treated patients with induced hypertension (mean arterial pressure > 110 mm-

Hg). Treatment was continued until normalization of follow-up imaging including CT- or MR-angiography.

Definition of CVS and DCI

CVS was suspected as any clinical worsening after securing of the aneurysm otherwise not explained. CVS was screened for with daily TCD ultrasound. At mean velocities > 160 cm/sec CVS was suspected and confirmation on CT-angiogram or DSA was strived for.

DCI was defined as any new ischemic lesion on the final CT or MRI before discharge compared to imaging 24 hours after aneurysm occlusion. Thus, lesions related to the procedure of aneurysm occlusion were ruled out. Imaging data was reviewed for the presence of CVS or DCI by an experienced neuroradiologist blinded to the results of MEA-testing.

Statistics

For the analysis of the demographic parameters of the included patients, Fisher's exact-test was used for categorical variables while the Mann Whitney U-test was used for continuous parameters. Normal distribution was tested using Shapiro-Wilk test. To analyze the results of the platelet count and results of MEA for changes over time, a repeated measures ANOVA with a Huynh-Feldt correction and Bonferroni-adjusted post-hoc analysis were performed. p-values < 0.05 were regarded as statistically significant. Analysis was performed with IBM SPSS 21.0 (IBM); Graphics were created using Prism 8 (GraphPad Software, LLC).

RESULTS

In total, 18 patients were included into this pilot study. CVS was diagnosed in 12 patients (66.7%); in five of these patients (27.8%) who developed CVS, DCI was detected. We compared patients without clinical or radiological evidence for CVS (no CVS) with patients who went on to develop CVS but no DCI (CVS+/DCI-) and DCI after being diagnosed with CVS (CVS+/DCI+). For baseline parameters such as age, gender, admission status (WFNS-grade), amount of blood as classified by the modified Fisher scale, aneurysm size, location, and treatment modality, we did not detect any significant difference between patients with CVS or DCI and those without such complications. A significant difference was observed regarding outcome classified by the mRS, which was significantly worse in patients with CVS and DCI (p = 0.047; Table 1).

When observing the overall patient population, the platelet count increased continuously after the ictus with a minimum on day 3. A significant increase in the overall platelet count compared to baseline was observed from day 8 onwards (p < 0.037, Figure 1a). The median platelet count exceeded the upper reference limit on day 10 and remained elevated thereafter. Analyzing each of the three study groups, we observed no significant increase in the median platelet count in patients of the no CVS group over the course of 14 days after the ictus (Figure 1b). Similarly, the observation of patients in the CVS+/DCI- and CVS+/DCI+ group did not show a significant increase in platelet count over the course of 14 days compared to baseline (Figure 1c and d). A repeated measures ANOVA was performed to assess differences in platelet count over time. Data were normally distributed, as assessed by the Shapiro-Wilk test (p < 0.05). Repeated measures ANOVA determined that mean levels of platelet count showed no statistically significant difference between measurements of the no CVS, the CVS+/DCI-, and the CVS+/DCI+ groups (*Fplatelets* (1.40, 4.21) = 3.64, p = 0.12).

Results of impedance aggregometry

The results of impedance aggregometry using TRAP-6 showed no statistically significant changes over the course of 14 days for all patients (Figure 2a). Furthermore, the analysis of the results of AUC_{TRAP} of the no CVS, CVS+/DCI-, and CVS+/DCI+ group revealed no statistically significant differences (Figure 2b - d). Data were normally distributed, as assessed by the Shapiro-Wilk test (p < 0.05). Repeated measures ANOVA determined that mean levels of AUC_{TRAP} detected no statistically significant difference between measurements of the no CVS, the CVS+/DCI-, and the CVS+/DCI+ groups ($F_{AUC_{TRAP}}$ (2.66, 7.98) = 1.06, p = 0.4).

Maximum platelet aggregation using ADP reagent showed a continuous increase over the 14-day observation period. AUC_{ADP} was consistently within the normal reference range (Figure 3a). There were no statistically significant differences in the results for the no CVS, CVS+/DCI-, and CVS+/DCI+ group (Figure 3b - d). Data were normally distributed, as assessed by the Shapiro-Wilk test (p < 0.05). Repeated measures ANOVA determined that mean levels of AUC_{ADP} detected no statistically significant difference between measurements of the no CVS, the CVS+/DCI-, and the CVS+/DCI+ groups ($F_{AUC_{ADP}}$ (2.63, 7.89) = 0.83, p = 0.50).

In the ASPItest, adding arachidonic acid, no statistical differences in platelet aggregation for all patients or groups of no CVS, CVS+/DCI-, and CVS+/DCI+ were found (Figure 4a - d), but AUC_{ASPI} remained below the normal range throughout the observation period of 14 days. Data were normally distributed, as assessed by the Shapiro-Wilk test (p < 0.05). Repeated measures ANOVA determined that mean levels of AUC_{ASPI} detected no statistically significant difference between measurements of the no CVS, the CVS+/DCI- and the CVS+/DCI+ groups ($F_{AUC_{ASPI}}$ (1.89, 5.69) = 0.63, p = 0.55). Bonferroni-adjusted post-hoc analysis also revealed no significant difference for platelet count and results of impedance aggregometry (AUC_{TRAP}, AUC_{ADP}, AUC_{ASPI}) for the subgroups of patients without CVS, the CVS+/DCI-, and the CVS+/DCI+ groups (data not shown).

Table 1. Demographic data.

Parameter	All patients (n = 18)	CVS+/DCI- (n = 12)	CVS+/DCI+ (n = 5)
Age (years [IQR])		ns	ns
	55 [46 - 66]	52 [46 - 65]	60 [50 - 72]
Female gender (n [%])		ns	ns
	13 [72]	10 [83]	4 [22]
BMI [SD]		ns	ns
	27.2 [3.9]	26.8 [3.6]	28.2 [4.9]
WFNS grade (n [%])		ns	ns
1	9 [50]	6 [50]	3 [60]
2	2 [11]	1 [8]	1 [20]
3	0	0	0
4	1 [5]	1 [8]	1 [20]
5	6	4 [33]	0
Modified Fisher grade (n [%])		ns	ns
0	1 [6]	1 [8]	1 [20]
1	2 [11]	0	0
2	4 [22]	2 [17]	0
3	5 [28]	4 [33]	1 [20]
4	6 [33]	5 [42]	3 [60]
Aneurysm size (mm [SD])		ns	ns
	7.3 [5.6]	6.0 [3.0]	5.6 [2.1]
Aneurysm site (n [%])		ns	ns
AComA	3 [17]	1 [8]	0
ICA	2 [11]	1 [8]	0
PCom	4 [22]	2 [17]	2 [40]
MCA	6 [33]	5 [42]	2 [40]
BA	2 [11]	2 [17]	1 [20]
Pericallosal	1 [5]	1 [8]	0
Aneurysm treatment (n [%])		ns	ns
Endovascular	12 [67]	8 [67]	3 [60]
Microsurgical	6 [33]	4 [33]	2 [40]
Outcome @ 6 months (n [%])		ns	p = 0.047
mRS 0-2	11 [61]	7 [58]	1 [20]
mRS 3-6	7 [39]	5 [42]	4 [80]

Patient characteristics. Data are given as means except for age which is presented as the median. Data comparisons were made with Fisher's exact test and Mann Whitney U-test where applicable. WFNS grade - admission status according to the grading scale suggested by the World Federation of Neurosurgical Societies. AComA - anterior communicating artery, ICA - internal carotid artery, PCom - posterior communicating artery, MCA - middle cerebral artery, BA - basilar artery, Pericallosal - pericallosal artery, endovascular - endovascular aneurysm treatment, microsurgical - microsurgical aneurysm treatment. Outcome rated according to the modified Rankin Scale (mRS). ns - not significant. IQR indicates interquartile range, SD standard deviation.

DISCUSSION

This is the first study to assess platelet aggregability as measured by MEA in patients after aneurysmal SAH. In this prospective study impedance aggregometric assessment of platelets showed no statistical differences in platelet aggregability for patients after SAH and irespective of the occurrence of CVS or DCI. Furthermore, our results of the study revealed a continuous increase in platelet count during 14 days after SAH and a signifi-

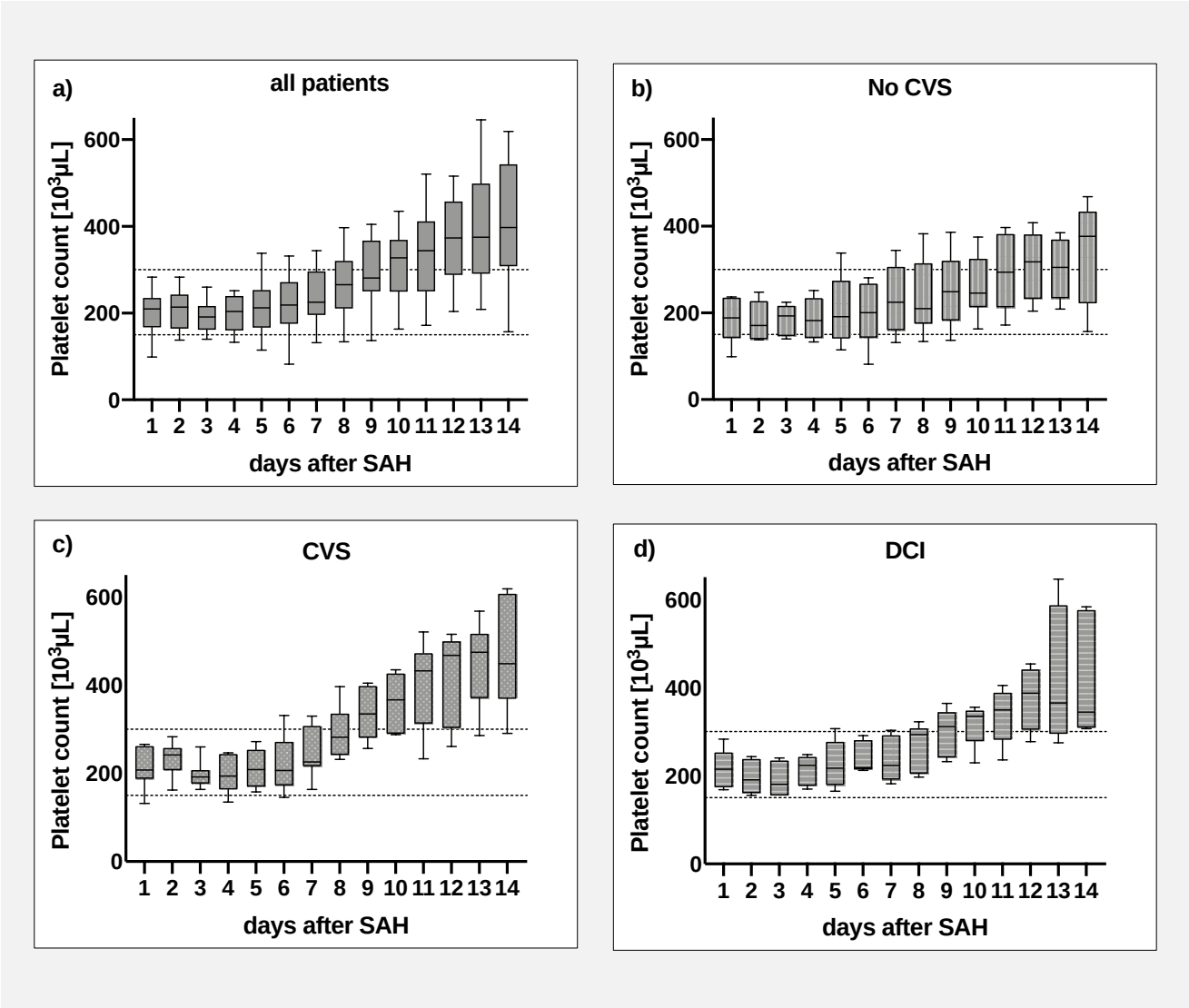

Figure 1. Platelet count in patients after SAH.

a) Box and whisker plots of platelet count after SAH of all patients. b) Box and whisker plots of platelet count after SAH without CVS. c) Box and whisker plots of platelet count after SAH and CVS. d) Box and whisker plots of platelet count after SAH and DCI.
The lower and upper bars represent the minimum and maximum, respectively. The lower and upper ends of the box represent the 25th and 75th percentiles, respectively; the line in the box represents the median. The dotted lines represent the lower and upper reference range.
SAH - subarachnoid hemorrhage, CVS - cerebral vasospasms, DCI - delayed cerebral ischemia, µL - microliters.

cantly worse outcome classified by the mRS in patients after SAH developing CVS and DCI.

To evaluate the role of platelet function in patients following SAH we performed impedance aggregometric measurements of aggregability. Our results showed a non-significant increase in platelet aggregability as measured by the TRAP-, ADP-, and ASPI-test within 14 days after SAH. We could not detect any significance, but a trend towards an early increase in platelet aggregability after SAH in AUC_{TRAP}. However, the results of our data revealed no statistically significant change in platelet function in groups of patients with CVS+/DCI- or CVS+/DCI+. Considering the clinically characteristic course of occurrence of CVS in patients after SAH [17], we detected no change in platelet aggregability after day 5. While the results of platelet activity in AUC_{ASPI} were predominantly detected below the normal reference range, results of AUC_{TRAP} and AUC_{ADP} were consistently in the normal reference range. Similarly, the aggregability of MEA as measured on admission was within the normal range in all patients and no significant difference in platelet aggregability could be demonstrated for groups of patients with CVS+/DCI- or CVS+/DCI+.

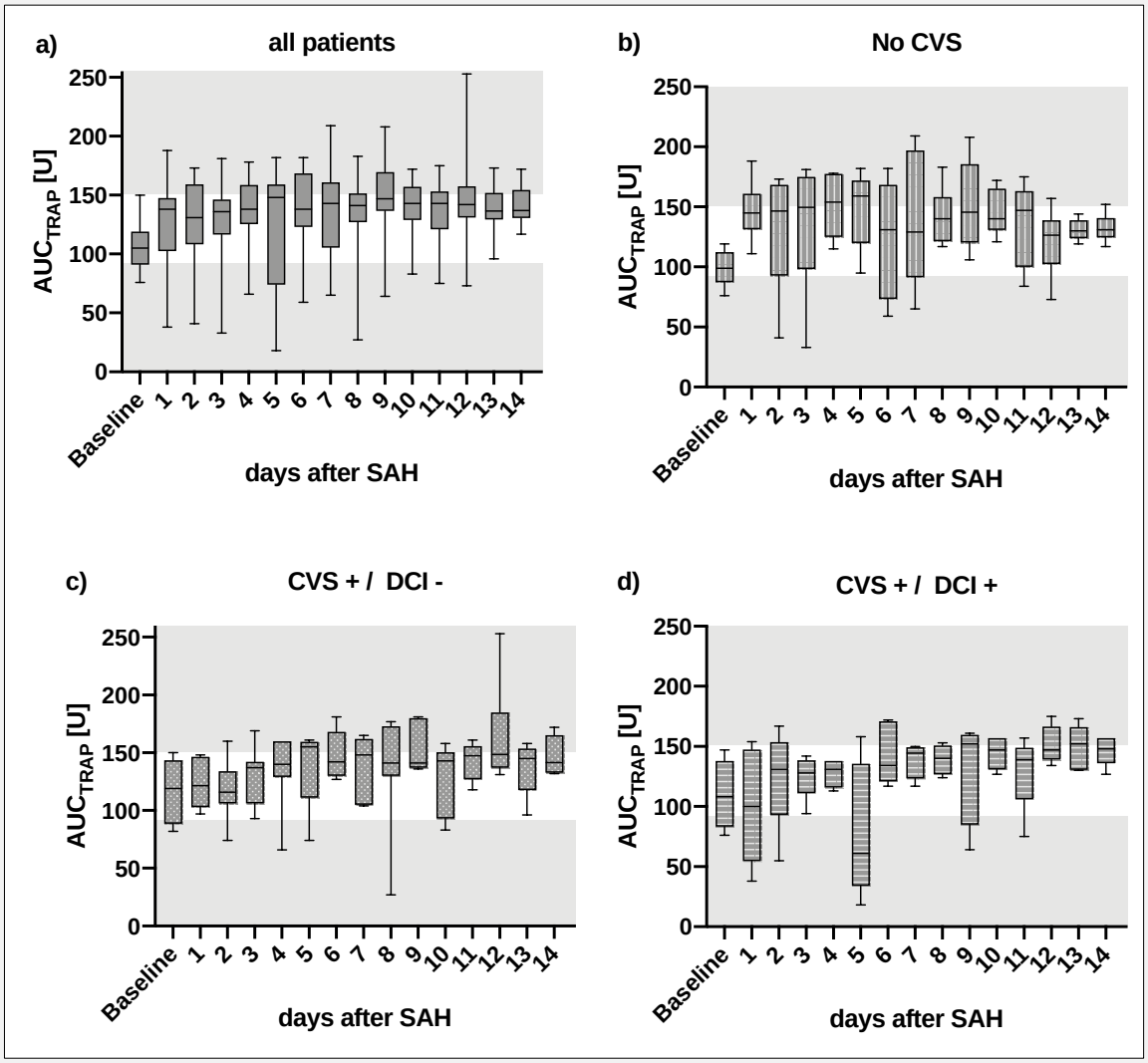

Figure 2. AUC_{TRAP} in patients after SAH.

a) Box and whisker plots of AUC_{TRAP} after SAH of all patients. b) Box and whisker plots of AUC_{TRAP} after SAH without CVS. c) Box and whisker plots of AUC_{TRAP} after SAH with CVS+/DCI-. d) Box and whisker plots of AUC_{TRAP} after SAH with CVS+/DCI+.
The lower and upper bars represent the minimum and maximum, respectively. The lower and upper ends of the box represent the 25th and 75th percentiles, respectively; the line in the box represents the median. The normal reference ranges of AUC_{TRAP} are highlighted in the white area.
TRAP - thrombin receptor activator peptide 6, AUC - area under the curve, U - units, SAH - subarachnoid haemorrhage, CVS - cerebral vasospasms, DCI - delayed cerebral ischemia.

As described earlier, some studies suggested a hypercoagulable state in patients with SAH, which might be caused by increased platelet function [18,19,7]. In this first and so far unique study using multiple electrode aggregometry, we could not confirm this hypothesis of an increased platelet aggregability in our observational study.

The potential influence of platelets leading to a hypercoagulable state after SAH in the development of CVS and/or DCI is still controversially discussed. As underlying mechanisms of platelet activation and aggregation, an increased secretion of factors such as P-selectin [20], platelet activating factor (PAF) [21], thromboxane [22] or platelet derived growth factor (PDGF-BB) [23] have been suggested. However, similar to our data, other studies reported an increase in platelet count [24,25].

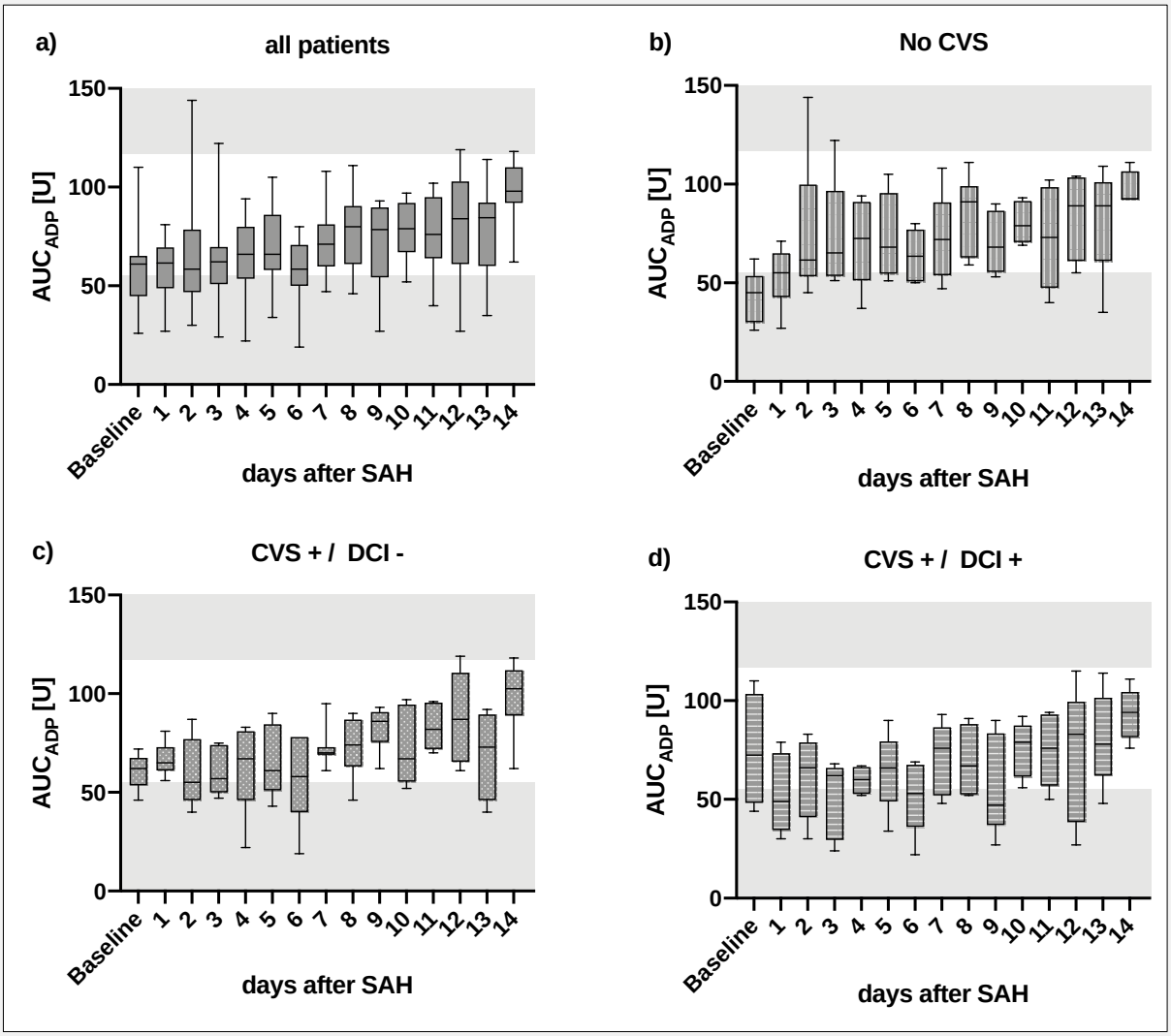

Figure 3. AUC_{ADP} in patients after SAH.

a) Box and whisker plots of AUC_{ADP} after SAH of all patients. b) Box and whisker plots of AUC_{ADP} after SAH without CVS. c) Box and whisker plots of AUC_{ADP} after SAH in patients with CVS+/DCI-. d) Box and whisker plots of AUC_{ADP} after SAH with CVS+/DCI+. ADP = Adenosine-5 diphosphate

The lower and upper bars represent the minimum and maximum, respectively. The lower and upper ends of the box represent the 25th and 75th percentiles, respectively; the line in the box represents the median. The normal reference ranges of AUC_{ADP} are highlighted in the white area.

AUC - area under the curve, U - units, SAH - subarachnoid hemorrhage, CVS - cerebral vasospasms, DCI - delayed cerebral ischemia.

Contrarily, Hirashima et al. found an early decrease in platelet count and a correlation with the occurrence of CVS and pointed out the important role of platelets in the pathophysiology of vasospasm after SAH [26]. In addition, they reported an increase in platelet count to a level higher than at admission. Schebesch et al. reported similar variations in platelet count with an initial decline and subsequent increase. However, the authors could not demonstrate a statistically significant correlation between platelet count and the occurrence of DCI [24]. Platelets might influence the occurrence of DCI not only by increasing the pure number. Experimental and clinical trials have suggested a platelet release of vasoactive factors, thus contributing to the development of CVS and DCI [27,20-22]. Pyne et al. suggested that both platelets and cerebrospinal fluid are required to

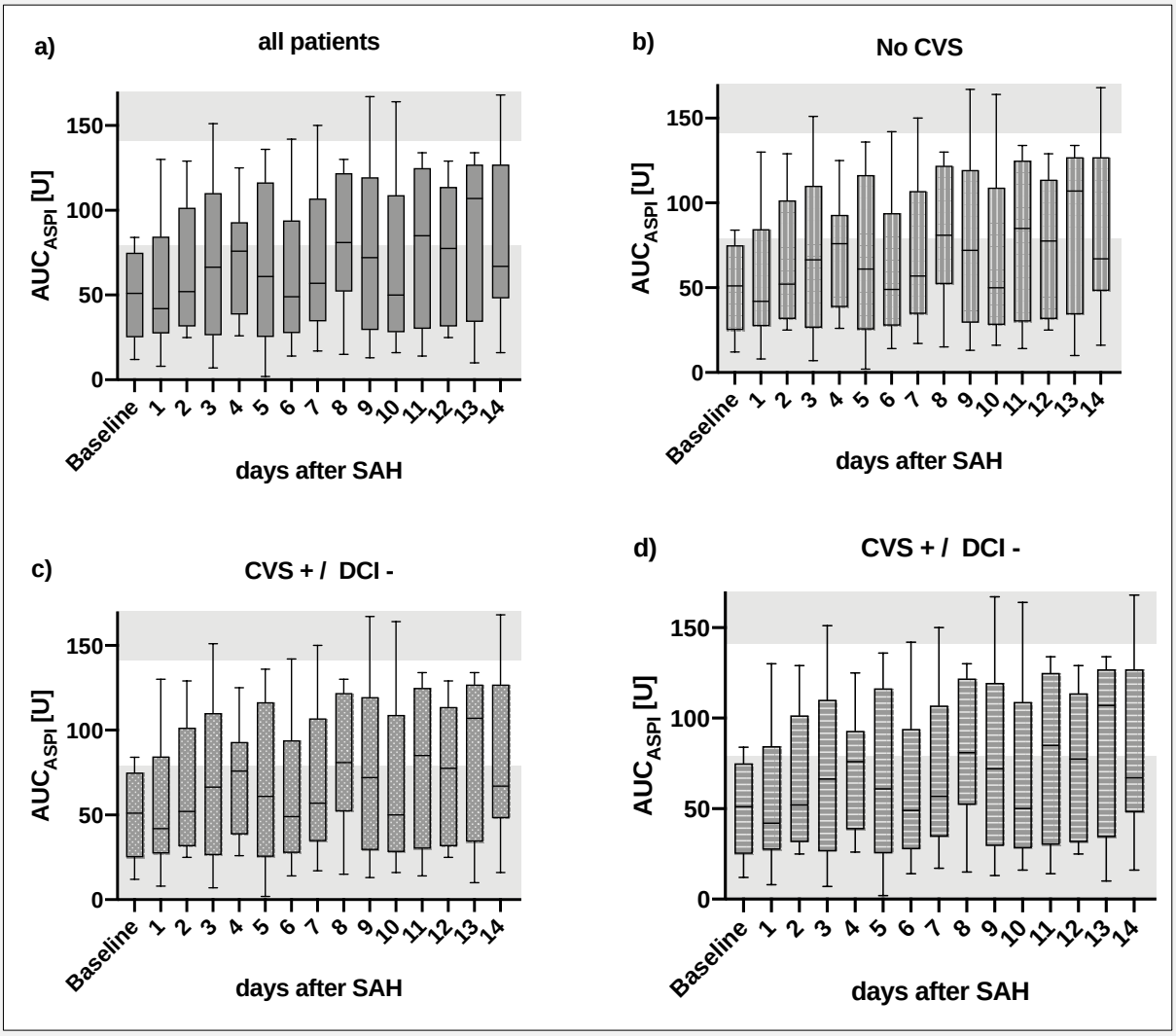

Figure 4. AUC_{ASPI} in patients after SAH.

a) Box and whisker plots of AUC_{ASPI} after SAH of all patients. b) Box and whisker plots of AUC_{ASPI} after SAH without CVS. c) Box and whisker plots of AUC_{ASPI} after SAH with CVS+/DCI-. d) Box and whisker plots of AUC_{ASPI} after SAH with CVS-/DCI-.
The lower and upper bars represent the minimum and maximum, respectively. The lower and upper ends of the box represent the 25th and 75th percentiles, respectively; the line in the box represents the median. The normal reference ranges of AUC_{ASPI} are highlighted in the white area.
ASPI - arachidonic acid, AUC - area under the curve, U - units, SAH - subarachnoid hemorrhage, CVS - cerebral vasospasms, DCI - delayed cerebral ischemia.

elicit CVS after SAH and that activated platelets might be contributing factors [27].

Nevertheless, some aspects must be considered when interpreting our results. Aneurysm-treatment alone might influence platelet activation as other studies reported that surgery profoundly influenced endothelial cell activation markers and concluded that subdivision of patients after aneurysm treatment is essential [20]. Due to the limited number of patients in this pilot study, further sub-analysis was not reasonable and therefore not performed. Moreover, an increase in the platelet count itself may be associated with enhanced platelet aggregability, as shown earlier for impedance aggregometric measurements [28,25]. Furthermore, pharmacologically induced hypertension might have an impact on platelet aggregability. Since it is standard practice to

pharmacologically induce hypertension at the time of clinical suspicion of CVS to prevent DCI [29], the increased shear force to which platelets are exposed as a result of high blood pressure could itself lead to platelet activation [30].

Based on our results and the concept of our study, we are unable to differentiate whether the observed trend of an increased platelet aggregability in patients after SAH is due to enhanced platelet activation, related to the increasing platelet count or might be influenced by pharmacological induction of hypertension in patients after SAH.

Limitations

Given the small sample size of our pilot study, the study may be underpowered and differences in aggregability between the groups analyzed may not have been detected.

Furthermore, there is no evidence that the suspected changes in platelet aggregability after SAH are due to pathophysiological changes that can be detected by multiple electrode aggregometry. As already mentioned in the discussion section, it is also conceivable that the increase in platelet count alone could lead to an increase in AUC of MEA results.

Concerning clinical characteristics, DCI may be more reliable than CVS due to its image-based nature, while CVS is often used as a mixed definition based on clinical and imaging data. As hypertension is known to influence platelet aggregability itself, this could also have an impact on measurements of platelet aggregability.

CONCLUSION

Besides an increase in platelet count, we detected no increase in platelet aggregability as measured by MEA in patients with aneurysmal SAH. Although we could not find significant differences in our pilot study with a limited number of patients, it is possible that an increased platelet aggregability may be present in patients after SAH, which may not be reflected by MEA.

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Declaration of Interest:

EA, CS, SY, BP, JK, VS, JP and HM have nothing to disclose. CW reports personal fees from CSL Behring, personal fees from Biotest, personal fees from Haemonetics, personal fees from Verum Diagnostica, personal fees from Roche, outside the submitted work.

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