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This is an accepted version of a paper published in *Scandinavian Journal of Clinical and Laboratory Investigation*. This paper has been peer-reviewed but does not include the final publisher proof-corrections or journal pagination.

Citation for the published paper:

Rodling Wahlström, M., Olivecrona, M., Koskinen, L., Naredi, S., Hultin, M. (2012)
"Subarachnoid haemorrhage induces an inflammatory response followed by a delayed
persisting increase in asymmetric dimethylarginine"
Scandinavian Journal of Clinical and Laboratory Investigation, 72(6): 484-489

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Subarachnoid haemorrhage induces an inflammatory response followed by a delayed persisting increase in asymmetric dimethylarginine

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Short title: ADMA, CRP and aneurysmal SAH

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Abstract

Object: Subarachnoid haemorrhage (SAH) is associated with an inflammatory systemic response and cardiovascular complications. Asymmetric dimethyl arginine (ADMA), an endogenous inhibitor of nitric oxide synthase, mediates vasoconstriction and might contribute to cerebral vasoconstriction and cardiovascular complications after SAH. ADMA is also involved in inflammation and induces endothelial dysfunction.

The aim of this study was to evaluate whether and how CRP (marker for systemic inflammation) and ADMA increased in patients during the acute phase (first week) after SAH. The ADMA level was also assessed in the patients in a non-acute phase (three months), and in healthy controls.

Methods: Prospective study of 20 patients with aneurysmal SAH. ADMA and CRP were followed daily during the first week after SAH and a follow up sample for ADMA was obtained three months later. A single blood sample for ADMA was collected from age and sex matched healthy controls (n=40, 2 for each case).

Results: CRP increased significantly from day 2; 16 (Confidence interval (CI) 10-23) mg/L to day 4; 84 (CI 47-120) mg/L, ($p<0.01$). ADMA increased significantly from day 2; 0.22 (CI 0.17-0.27) $\mu\text{mol/L}$, to day 7; 0.37 (CI 0.21-0.54) $\mu\text{mol/L}$, $p<0.01$. ADMA remained elevated at a three-month follow-up 0.36 (CI 0.31-0.42) $\mu\text{mol/L}$.

ADMA in the first sample from the patients (day 1-3); 0.25 (CI 0.19-0.30) $\mu\text{mol/L}$, was not different from ADMA in matched healthy controls; 0.25 (CI 0.20-0.31), $p>0.05$.

Conclusion: After SAH, CRP and ADMA in serum increased significantly during the first week and ADMA remained elevated three months later.

Keywords: Acute-Phase Reaction, C-Reactive Protein, Subarachnoid Hemorrhage, Intracranial Aneurysm, Arginine/administration & dosage/analogs & derivatives/blood

Introduction

Asymmetric dimethyl arginine (ADMA) is an endogenous competitive inhibitor of nitric oxide synthase (NOS). Increased concentration of ADMA decreases the availability of nitric oxide (NO) and thus induces vasoconstriction. ADMA is also associated with inflammation and can cause endothelial dysfunction [1-4]. ADMA is an independent risk factor for vascular disease and is elevated in various clinical conditions such as atherosclerosis, coronary artery disease, stroke, chronic renal disease, critical illness and preeclampsia [1,3-8].

Subarachnoid haemorrhage (SAH) from a ruptured cerebral aneurysm is associated with systemic cardiopulmonary complications and cerebral vasospasm, contributing substantially to morbidity and mortality [9-11]. A massive systemic inflammatory response is triggered by SAH and induces an increase in inflammatory markers such as C-Reactive Protein (CRP) [12]. High ADMA concentrations after ischemic stroke has been associated to un-favourable outcome, and elevated concentrations of ADMA can be lowered by anti-inflammatory treatment with statins [13,14]. However, the timing of ADMA and CRP as markers for inflammatory response after SAH remains to be elucidated [15].

In a primate model of SAH, ADMA in cerebrospinal fluid (CSF), but not in serum, was shown to be elevated 7 and 14 days after SAH [16]. In a clinical study of patients with aneurysmal SAH, a strong correlation between ADMA concentrations in CSF and degree of cerebral vasospasm was found, but ADMA concentrations in serum remained unchanged during the study period [17]. This is in contrast to the rapid increase in plasma concentrations of ADMA after ischemic stroke reported by Worthmann et al [14]. In a small study on patients with SAH caused by aneurysmal bleeding or trauma, increased concentrations of ADMA were shown both in both plasma and CSF [18].

The primary aim of this study was to evaluate whether and when CRP and ADMA in serum increased in patients during the acute phase (first week) after aneurysmal SAH. ADMA was also assessed in the patients in a non-acute phase (three months) after SAH and compared with sex and age matched healthy controls.

Patients and Methods

This is a prospective study of 20 patients with SAH due to a ruptured cerebral aneurysm. All patients were treated at the neurosurgical department at Umeå university hospital, Sweden. The aneurysm was verified by CT-angiography or digital subtraction angiography. The intention was that endovascular coiling or surgical clips ligation should be performed within 24 hours after arrival to the university hospital. Due to logistic reasons, only patients arriving Monday-Friday were included in the study. The documented time for SAH was either the exact time or, if the patient was found unconscious and the exact time was unknown, an approximated time as close as possible. Day one was defined as the first 24 hours after the first clinical sign of SAH. Blood samples for CRP was obtained daily with the clinical morning laboratory samples and blood samples for ADMA analysis were collected daily, 9-11 am, for seven consecutive days. The serum samples were centrifuged after 15 minutes of resting time; supernatants were collected, and instantly frozen and stored at -80°C. C-reactive protein (CRP) was measured at the accredited Umeå University Hospital laboratory using standard, fully automated, laboratory procedures.

Upon arrival medical history was obtained and a physical examination was performed. Three scoring systems were used; the Glasgow Coma Scale (GCS) for assessment of consciousness, the Hunt-Hess grade (H&H) for evaluation of the severity of SAH and the Fisher score for estimation of the degree of haemorrhage at the first CT-scan [19]. The patients were treated according to a local protocol for SAH, which included normoventilation, normovolemia and keeping glucose, sodium, haemoglobin and albumin within normal limits. Prophylactic treatment with continuous infusion of nimodipine (Nimotop®, Bayer) 0.2 mg/mL, 0-15 mL/h intravenously (iv.), with a target rate of 10 mL/hour, was used for prevention of delayed ischemic neurologic deficits. The infusion was started almost without delay after arrival.

Transcranial Doppler (TCD) for evaluation of blood flow velocity was performed if cerebral vasospasm was clinically suspected. TCD verified cerebral vasospasm was defined as a flow velocity

>120 cm/s in the middle cerebral artery. Physiological parameters (systemic blood pressure, heart rate, oxygen saturation) were continuously monitored (Marquette, GE Medical Systems AB, Stockholm, Sweden).

At the three-month follow-up, a blood sample for ADMA determination was collected and independent research nurses assessed the neurological outcome according to the Glasgow Outcome Scale (GOS).

Reference values for ADMA was obtained by analysing serum samples from age and sex matched patients from a regional health study project, the MONICA-project [20]. For each patient, two age and sex matched control patients were randomly selected. All samples in the Monica Cohort are fasting samples obtained daytime.

The Research Ethics Committee of Umeå approved the study protocol. Written informed consent was obtained from the patient or next of kin.

ADMA analysis

All reagents and chemicals for the analysis of ADMA were from Sigma-Aldrich (Stockholm, Sweden).

To minimize the risk for analysis bias, all ADMA samples were analysed in batch after the collection of samples was finished. To analyze ADMA, basic compounds were separated on an Oasis MCX μ Elution plate (Waters, Milford, MA, USA) by serial washings with acidic and neutral washings before the basic compounds were washed out from the anion-exchange column by with ammonium hydrochloride/distilled water/methanol (10:40:50) [21]. Asparagine was used as internal standard (5 μ mol/L). The elute was dried, dissolved in water, derivatized with OPA, and separated on HPLC with an isocratic solution of 80 % buffer A (0.200 M Sodium Acetate, pH 7.20:water: methanol, 462:1488:100) and 20 % pure methanol on a ACE 3 C18 column 3x100 mm (HiChrom, Berkshire, UK). ADMA and the internal standard homoarginine were detected using a fluorescence detector (Waters M470, Millipore Corp, Milford, MA, USA). Between each patient in the batch run a standard curve and two standard samples were included to ensure the stability of the HPLC method. Integration of area under the curve for each peak was done using Chromeleon Software (Dionex Corporation, Sunnyvale, CA, USA). The precision of the HPLC assay was 3.8 % at the concentrations present in serum.

Statistics

GraphPad Prism ver 5.0 for Mac (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis. Due to a skewed normal distribution, non-parametric tests were used for testing statistical significance. For comparison of ADMA the day after SAH (day 2) vs. controls, Mann-Whitneys test was used. For paired test of ADMA at day 7 vs three months, Wilcoxon signed-rank matched pairs test was used. For comparison of ADMA and CRP at different time-points during the first week, Kruskal-Wallis test with Bonferroni-Dunn's multiple comparison test for post-hoc analysis was used. $p < 0.05$ was considered statistically significant. Data are presented as mean $\pm$ SD, mean and confidence interval (CI) or median (range).

Results

Study population

Twenty patients with aneurysmal SAH, 12 men and 8 women, were included in the study. Basic characteristics of the study population are given in Table 1. All patients were treated with i.v. nimodipine (0.2 mg/mL, 6.0 ± 3.7 mL/hour).

ADMA concentrations in acute phase in patients and compared with healthy controls

Upon arrival at the university hospital, 75% within 24 hours of the initial sign of SAH and the remainder within 25-48 h, ADMA serum concentrations in patients were not significantly different in age and sex matched healthy controls; 0.25 (CI 0.19 - 0.30) $\mu\text{mol/L}$ in patients, compared with 0.25 (CI 0.20 - 0.31) $\mu\text{mol/L}$ in controls, $p > 0.05$. During the first week after SAH, ADMA increased with 68% from day 2; 0.22 (CI 0.17 - 0.27) $\mu\text{mol/L}$ to day 7; 0.37 (CI 0.21 - 0.54) $\mu\text{mol/L}$, $p < 0.01$) (Fig 1a).

Fig 1. ADMA and CRP in serum.

Concentrations of ADMA (panel a) and CRP (panel b) after SAH. Data are presented as mean $\pm$ confidence interval (CI). n indicates number of patient samples at each time-point. * and ** denotes statistically significant difference from day 2 after SAH ($p < 0.05$ and $p < 0.01$).

ADMA and different clinical features

Treatment with surgical ligation or endovascular coil was performed day 2 (median; range 1-6) after SAH. No significant difference was found in ADMA between the two treatment groups at day 7; endovascularly coiled 0.40 (CI 0.13-0.67) $\mu\text{mol/L}$ vs surgically ligated 0.33 (CI 0.23-0.43) $\mu\text{mol/L}$, $p > 0.05$.

Only two patients had clinical and/or TCD verified vasospasm, none of these patients showed any extreme values of ADMA. Eleven of the patients were smokers before the SAH. There were no differences seen between smokers or non-smokers in ADMA concentrations in the acute or non-acute phase ($0.16 < p < 0.76$ for the various days).

Nine patients had pathological ECG on admission to hospital. Four of the patients had pathologically increased troponin T and one patient had ST-elevation infarction ($V2 > 2 \text{ mm}$) during the first week after SAH. ADMA did not differ significantly between patients with ($n=5$) and without ($n=15$) signs of myocardial infarction (Day 2; 0.22 (CI 0.15–0.29) $\mu\text{mol/L}$ without vs 0.21 (CI 0.13-0.30) $\mu\text{mol/L}$ with signs of myocardial infarction, $p > 0.05$; Day 7; 0.39 (CI 0.16-0.62) $\mu\text{mol/L}$ without vs 0.32 (CI 0.21-0.42) $\mu\text{mol/L}$ with signs of myocardial infarction, $p > 0.05$).

CRP as marker for inflammation in acute phase

CRP increased from day 2 to day 4-7 after SAH (Fig 1b). The increase in CRP appears to be earlier than the increase of ADMA (compare panel a and b in Fig 1). No differences were seen between smokers and non-smokers in CRP after SAH ($0.33 < p < 0.72$ for the various days).

ADMA concentrations in chronic phase

Of the 20 patients included in the study, 15 came to the follow-up visit three months after the SAH.

Three months after SAH, ADMA in serum was still elevated and remained at the same level as day 7 in the acute phase. The difference between ADMA day 2 in the acute phase, 0.25 (CI 0.19-0.31) $\mu\text{mol/L}$, compared with ADMA three months after the SAH ($n=15$); 0.36 (CI 0.31-0.42) $\mu\text{mol/L}$, was significant ($p < 0.01$). There was no significant difference in ADMA from day seven in the acute phase ($n=19$); 0.37 (CI 0.21-0.54) $\mu\text{mol/L}$ compared with ADMA three months after the SAH ($n=15$); 0.36 (CI 0.31-0.42) $\mu\text{mol/L}$, $p > 0.05$.

The GOS score was 5 (median; range 1-5) at the three-month follow-up. Two patients died during the study (at day 7 and day 34) and three patients with GOS 3 did not show up. There was no difference in ADMA day 7 between patients with favourable outcome (GOS 4-5; ADMA 0.39 (CI 0.14-0.64) $\mu\text{mol/L}$) compared with unfavourable outcome (GOS 1-3; ADMA 0.33 (CI 0.24-0.42) $\mu\text{mol/L}$), $p > 0.05$.

Table 1. Basic characteristics

Sex M/F		
(n)		12/8
Age (years)		
mean±SD		60±8
Score		
median (range)		
	GCS ¹	9 (3-15)
	H&H ²	3 (1-4)
	Fisher ³	4 (3-4)
Medical history⁴		
n (%)	smokers	11/20 (55)
	hypertension	10/20 (50)
	hyperlipidemia	4/20 (20)
	angina pectoris	3/20 (15)
	stroke	2/20 (10)
Aneurysm location⁵		
n (%)	ACoA	7/20 (35)
	MCA	6/20 (30)
	PICA	2/20 (10)
	ICA	2/20 (10)
	BA	2/10 (10)
	PCoA	1/20 (5)
Treatment		
n (%)	Surgical ligation	7/20 (35)
	Endovascular coil	12/20(60)
	No treatment	1/20 (5)

¹Glasgow coma scale; extends from 15; fully awake, to 3; deep coma

²Hunt&Hess scale; Hunt and Hess classification, extends from 1; minimal symptoms, to 5; deep coma

³Fisher score; amount of SAH haemorrhage from 1; no blood detected, to 4; intracerebral or intraventricular haemorrhage

⁴One patient can have more than one diagnosis

⁵ACoA = anterior communicating artery, MCA = middle cerebral artery, PICA = posterior inferior cerebellar artery, BA = basilar artery, ICA = internal carotid artery, PCoA = posterior communicating artery

Discussion

There were three main findings in the present study; (1) CRP as a marker for inflammation increased significantly during the first week after SAH, (2) ADMA in serum increased significantly during the first week after aneurysmal SAH, and (3) ADMA in serum remained elevated three months later.

CRP increased significantly during the acute phase after SAH. This elevation of CRP came earlier than the increase in ADMA, and it is tempting to speculate that CRP and ADMA are markers for different phases in the inflammatory response. However, this study was not designed to allow for testing this hypothesis.

The novelty in this study is that ADMA in serum increased by 68% during the first week after aneurysmal SAH. A previous study on aneurysmal SAH and ADMA in humans failed to show an increase in blood, only elevations of ADMA in CSF was detected [17]. In a small study with a case mixture of SAH caused by both cerebral aneurysms and trauma, an increase in systemic ADMA was detected [18]. In the present study, the increase of ADMA was somewhat delayed compared with the increase of CRP and did not reach significance until day 6. The time-frame for ADMA elevation was delayed compared with the immediate increase seen in norepinephrine after SAH concomitantly with the development of early cardiovascular complications [22].

The increase of ADMA in the acute phase correlated more with the time period for occurrence of cerebral vasospasm after SAH [23]. Despite relatively high Fisher and H&H grades in our patients, which might indicate an increased risk for cerebral vasospasm, only 2/20 patients developed a verified cerebral vasospasm. However, this study was neither designed to confirm any association between ADMA and cerebral vasospasm nor to confirm an association between ADMA and cardiovascular performance. There was a low incidence of clinically detected cerebral vasospasm, but TCD was only performed when cerebral vasospasm was clinically suspected. The used treatment protocol focusing on normovolemia and the use of intravenous nimodipine might also have contributed to the low incidence [24].

The second novel finding in this study was that ADMA remained elevated three months after SAH. The clinical implication of this chronic elevation of ADMA is unclear. However, after SAH, ultra-structural changes has been found in cerebral arteries [25] and increased carotid-artery intima-media thickness has been linked to increased ADMA concentrations [25,26]. Whether ADMA is directly involved in these processes is unknown, but elevated ADMA have been linked to numerous cardiovascular diseases [4,8].

Atherosclerosis and inflammation are tightly linked and elevated CRP have clear prognostic value after major cardiovascular events [27]. In a small study on patients with acute liver failure, a tight correlation was shown between pro-inflammatory cytokines and ADMA [28]. The degradation of ADMA in the liver [29] makes the interpretation of that study difficult. However, the relationship between inflammation, as indicated by elevated CRP, and endothelial dysfunction, as measured by ADMA, might hold a key to minimizing the consequences for the patient after surviving aneurysmal SAH.

The elevation of ADMA found in this study might be a specific reaction after SAH, but it is tempting to suggest that the ADMA-elevation is unspecific and triggered by a generalized inflammatory activation. An increased risk of cardiovascular death has been reported in SAH patients [30]. An interesting question is whether SAH by a combination of stress and inflammatory response can induce a persisting endothelial dysfunction, indicated by elevated ADMA concentration?

Limitations of the study; ADMA and CRP in serum was not followed long enough to follow the drop in serum concentrations and not frequent enough to allow for a detailed analysis of the relation between the increases in ADMA and CRP. The low number of patients (20) included in the study makes subgroups analysis difficult. A thorough analysis of smoking versus ADMA and CRP after SAH was not a primary aim of this study. To make a detailed comparison of the relation between ADMA, CRP and cerebral vasospasm, repeated cerebral angiographies would have been needed. In the present study only clinically detectable cerebral vasospasm was followed.

Conclusion

This study shows that ADMA in serum increased during the first week after aneurysmal SAH and remained elevated for at least three months. The relationships and interdependencies between ADMA concentrations in serum, systemic inflammation and cerebral vasospasm are interesting and warrant further studies.

Conflict of interest

The authors of this article have no conflict of interest to disclose.

Acknowledgements and funding

We express our gratitude to the research nurses at the Neurosurgical department for their invaluable enthusiasm and quality of work.

This study was supported by grants from the Swedish Medical Society, the Neurological Foundation at Umeå University and the Faculty of Medicine at Umeå University, Sweden.

Previous presentation

Parts of the results were presented as an abstract at ESCIM 2006, September 24-27, Barcelona, Spain.

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