Impaired endothelial function after aneurysmal subarachnoid haemorrhage correlates with arginine:asymmetric dimethylarginine ratio†

A. Bergström1, J. M. Staalsø3, B. Romner2 and N. V. Olsen1,3*

1 Department of Neuroanaesthesia and 2 Department of Neurosurgery, The Neuroscience Centre, Copenhagen University Hospital (Rigshospitalet), Copenhagen, Denmark
3 Department of Neuroscience and Pharmacology, The Health Faculty, University of Copenhagen, Copenhagen, Denmark
* Corresponding author. E-mail: nvolsen@sund.ku.dk

Editor’s key points
- Endothelial dysfunction might contribute to cerebral vasospasm after subarachnoid haemorrhage (SAH).
- Reactive hyperaemia index (RHI) measured by peripheral arterial tonometry was reduced early after SAH, and correlated with 30 day mortality.
- Early endothelial dysfunction after SAH detected by RHI warrants further study to predict treatment and outcome.

Background. Endothelial dysfunction might be involved in the development of cerebral vasospasm after aneurysmal subarachnoid haemorrhage (SAH).

Methods. This prospective observational study of 48 SAH subjects and 23 control subjects examined associations between reactive hyperaemia index (RHI) measured by peripheral arterial tonometry and plasma concentrations of S-100B protein, nitrite/nitrate, arginine, and asymmetric dimethyl arginine (ADMA). Clinical variables were flow velocity in the middle cerebral artery ($V_{MCA}$), angiographic vasospasm, delayed neurological deficit, and 30 day survival. Five consecutive measurements were obtained at days 0–2, 3–5, 6–8, 9–11, and 12–15.

Results. RHI was 1.67 (0.46) at days 0–2 after SAH but increased at days 3–15 to the same levels as in controls ($P<0.05$ compared with days 0–2). RHI was lower in subjects who died before day 30 ($P=0.07$), but no trends were observed in relation to angiographic vasospasm or delayed neurological deficit. Both arginine and ADMA increased after SAH compared with days 0–2 ($P<0.05$). S-100B was highest in non-survivors ($P<0.01$) and in subjects with neurological deficit ($P<0.01$). A positive correlation was found between RHI and arginine:ADMA ratio ($r=0.43$, $P<0.005$), but not with nitrite/nitrate, $V_{MCA}$, or S-100B.

Conclusions. Peripheral flow-mediated vasodilation is attenuated in the first days after SAH indicating acute systemic endothelial dysfunction. Impairment of endothelial function after SAH correlates with imbalance of the arginine/ADMA pathway.

Keywords: ADMA; arginine; endothelial dysfunction; S-100B; subarachnoid haemorrhage

Accepted for publication: 8 July 2013

Acute aneurysmal subarachnoid haemorrhage (SAH) is one of the most devastating neurological diseases. Delayed cerebral ischaemia from vasospasm is a common complication of SAH and is associated with poor clinical outcome.1 Cerebral vasospasm can occur several days after the initial haemorrhage and it remains difficult to predict outcome.2 3 In SAH, nitric oxide (NO) from endothelium, autonomic nerves, and neurones is crucial for the regulation of cerebral blood flow.4 Numerous studies have demonstrated that endothelial dysfunction in coronary and peripheral vessels is associated with increased risk of ischaemic heart disease and stroke.5 7 8 Endothelial dysfunction contributes to development of cerebral vasospasm after SAH,6 but it is unknown whether it can be detected in peripheral vessels.

Assessment of endothelial function has largely been done by evaluation of flow-mediated vasodilation in the brachial artery with ultrasound.6 The importance of endothelial dysfunction for the development of vascular disease is well described,2 8 but assessment has been limited by methodological problems including a lack of standardization and great observer variability.2 8 The introduction of a new method based on amplitude tonometry in the fingertip (peripheral arterial tonometry, PAT) allows easy, quick, and standardized measurement of endothelial function.5 8 In a large clinical trial, PAT was reported to have reliable reproducibility.8 Studies demonstrate a correlation between cardiovascular risk factors and the PAT hyperaemic response.8 The method is consistent with flow-mediated
vasodilatation in the brachial artery, and experiments with NO donors and NO synthase inhibitors indicate that the PAT signal, in part, is NO-dependent.\(^8\) In severe acute conditions such as sepsis and major surgical operations, measurements of endothelial function might be applicable in prospective risk stratification of patients.\(^9\)\(^10\) Measurement of peripheral vasodilator response as an expression of endothelial dysfunction correlate with outcome,\(^11\)\(^12\) and it has been shown that the PAT signal can be used as a user-independent risk factor for development of late complications after an acute coronary syndrome.\(^11\) In SAH patients, the measurement might help identify patients at risk for developing life-threatening cerebral vasospasm.

We hypothesized that the results of early measurements of endothelial function with PAT correlate with other indices for the development of cerebral vasospasm, that is, transcranial Doppler (TCD) measurements of velocities in large cerebral arteries, and plasma concentrations of nitrite/nitrate, S-100B, arginine, and asymmetric dimethylarginine (ADMA). We also tested whether measurements with PAT correlate with 30 day mortality after SAH.

**Methods**

**Subjects**

Forty-eight patients admitted to the neurointensive care unit at the Copenhagen University Hospital between February 2011 and December 2011 with spontaneous aneurysmal SAH verified by computed tomography (CT) were studied prospectively in the 30 days after the initial rupture of the aneurysm. The presence of an aneurysm was confirmed by CT angiography, digital subtraction angiography, or during surgery. Exclusion criteria included prior spontaneous SAH, age <18, or pending cerebral herniation. Control subjects (n=23) were recruited with no history or clinical signs of cancer, diabetes mellitus, arterial hypertension, cerebral disease, or current smoking. The study population is a subpopulation of a previously published study; of which, we have published serum nitrite/nitrate concentrations and plasma arginine:ADMA ratios.\(^12\)

**Ethics**

Written informed consent was obtained from each participant or by the nearest relative and the patient’s general practitioner. The study protocol was approved by the Regional Ethical Committee of Copenhagen Region (H-3-2010-136).

**Data acquisition**

Data on prior medical history and medicine consumption were obtained from the SAH patients or their nearest relatives. In SAH patients, measurements were grouped into five consecutive intervals with 3 days in each interval: days 0–2, days 3–5, days 6–8, days 9–11, and days 12–14. The use of vasoactive drugs (remifentanil, propofol, and/or norepinephrine) was noted in each testing period.

**Measurement of peripheral endothelial function**

Peripheral endothelial function was assessed by non-invasive digital pulse amplitude tonometry by the use of an EndoPAT 2000 (Itamar Medical Ltd, Caesarea, Israel). The system collects data digitally, removes noise, and performs semi-automatic calculation of the post-occlusion/pre-occlusion ratio, the reactive hyperaemia index (RHI). The device consists of two fingertip probes measuring volume changes in relation to arterial pulsation. An air-filled chamber in the fingertip probe provides a uniform pressure preventing the venoarterial vasoconstrictor reflex. Measurements were performed on both arms simultaneously to account for systemic changes in vascular tone.

The SAH subjects underwent bedside EndoPAT measurements in the supine position at study inclusion which was carried out as soon as possible after SAH, and then four more times within a 14 day period. After a 5 min equilibration period, a cuff was rapidly inflated on one upper arm up to 200 mm Hg, or to 50 mm Hg above systolic arterial pressure, in 5 min according to recommendations for maximal response.\(^13\) Reactive hyperaemia was induced when the cuff was released. A similar measurement was performed in the control subjects after 15 min of sitting at rest.

**Cerebral vasospasm**

Neurological condition was evaluated by the World Federation of Neurosurgical Societies grading scale for SAH.\(^14\) A decrease in Glasgow coma score of 2 points or development of major focal deficits between days 3 and 12 was termed delayed neurological deficit. TCD measurements of the mean flow velocity in the middle cerebral artery (V\(_{\text{MCA}}\)) were performed bilaterally in duplicate with colour-coded duplex ultrasound (Micromaxx, Sonosite Ltd, Hitchin, UK; P17/5-1 MHz probe). Cerebral vasospasm was defined as a V\(_{\text{MCA}}\) > 200 cm s\(^{-1}\) or a difference between sides of >50 cm s\(^{-1}\) occurring between days 3 and 12 (both included) after SAH. In three subjects, it was not possible to obtain a reliable TCD signal. Cerebral angiographic vasospasm was defined as clinical symptoms in combination with a positive angiogram. Patients were subjected to angiography only on indication by the treating clinician.

**Laboratory assays**

Blood samples were collected immediately before the EndoPAT measurements and were stored at −80°C until analysis. Samples for measurement of nitrite/nitrate were centrifuged through a 30 kDa micropore filter (Nanosep 30k Omega, PALL Corp., Ann Arbor, MI, US) before duplicate analysis with a commercially available NO\(_x\) detection kit based on the Griess reaction (cat. 780001, Nitrite/Nitrate Colorimetric Assay Kit, Cayman Chemicals, Ann Arbor, MI, USA). Concentration of S-100B was measured by ELISA (Roche Diagnostics, Mannheim, Germany). Arginine and ADMA were measured with high performance liquid chromatography as previously described.\(^12\) Briefly, solid phase extraction of plasma samples (Oasis MCX columns, Waters, Milford, MA, USA) was followed by derivatization of basic amino acids with o-phthalaldehyde to form fluorescent compounds. These were separated on a Symmetry C18 column (Waters) with a flow rate of 1.1 ml min\(^{-1}\) (Shimadzu LC-20AD pumps, Kyoto, Japan) and measured on a fluorescence detector (Shimadzu RF-20A). Monoethyl arginine was used as the internal standard.
standard. The peak area was quantified manually on a computer with Empower 2.0 software (Waters).

Statistical analysis

Statistical analysis was performed with R-2.15.1 software for Mac, and additional packages nlme and ggplot2. Student's t-test was used to compare RHI between control and SAH subjects at baseline. Longitudinal measurements of variables were analysed using a linear mixed effects models (nlme package) to account for within-subject correlation of serial measurements. The subject term entered the model as a random effect on the intercept. Model assumptions were checked by residual plots. Values are given as mean (sd).

Results

Data from 48 SAH patients and 23 control subjects entered the analysis. Subject characteristics are presented in Table 1. SAH subject years range from 22 to 81. Control subject age range from 28–64 yr (P = 0.00005 compared with patients). Body mass index (kg m⁻²) in SAH and control subjects ranged 18–34 and 18–26, respectively (P = 0.18). Notable characteristics of this group of SAH subjects are a higher than usual prevalence of female sex and smoking. Aneurysms in the anterior complex or the middle cerebral artery were most frequent (Table 1), which is comparable with previous findings in a Danish population. The 30 day mortality was 15% (Table 1).

Measurements with EndoPAT were feasible in almost all subjects. All five planned EndoPAT measurements were not performed in all subjects. In 33 of the 48 subjects, it was possible to perform an EndoPAT measurement. Reasons for not being measured five times were late hospitalization, death before day 14, transfer to another hospital or early discharge, and inability to cooperate. Only two subjects were unable to cooperate on all five occasions. In one control subject, it was impossible to obtain a pulse wave reading.

The RHI in control subjects was 1.81 (0.37). In SAH subjects, EndoPAT measurements increased by 0.24 (Pmixed-model t-test = 0.02, CI95: 0.04 – 0.44) from days 0–2 to days 3–5 and remained stable thereafter (Table 2). The difference between days 0–2 and days 6–8 was 0.23 (Pmixed-model t-test = 0.024, CI95: 0.03 – 0.43). The RHI in control subjects was 0.15 higher than RHI days 0–2 in SAH subjects (P = 0.036), the difference diminished to −0.05 when comparing controls with RHI days 6–8 (P = 0.60, CI95: −0.29 to 0.17).

TCM measurements were performed in 36 subjects. In the remaining five subjects, TCM measurements were impossible to perform for technical reasons. Positive findings of vasoconstriction were found in 21 out of 36 subjects. V_mca by average tended to increase (Table 2). CT-angiography was performed in 18 subjects and positive findings of vasospasm were found in 11. Overall, there was no association between V_mca and RHI.

On average, S-100B decreased through the observation period, but only values on days 10–12 were statistically different compared with baseline (Table 2). Plasma levels of nitrite/nitrate remained unchanged (Table 2). Plasma arginine increased from days 0 to 2 onwards (Table 2). Also ADMA slightly increased after SAH; thus, the arginine:ADMA ratio significantly increased throughout the observation period (Table 2).

Serial data of RHI in relation to 30 day survival, delayed neurological deficit, TCD-vasospasm, and angiographic vasospasm are presented in Figure 1. RHI was lower on days 9–12 (CI95: −1.09 to 0.04, Pmixed-model t-test = 0.07) and days 13–15 (CI95: −1.03 to 0.07, Pmixed-model t-test = 0.08) in subjects not surviving to day 30. When RHI was averaged separately for technical reasons, the Cox proportional hazards model showed an association between low RHI in week 2 and mortality (P = 0.036). A decrease in RHI by 0.5 corresponded to a hazard ratio of 3.3 (CI95 = 1.0–11). The week 1 average RHI was not associated with mortality (P = 0.51). There were no trends towards differences in RHI according to delayed neurological deficit, TCD vasospasm, or angiographic vasospasm.

Subjects receiving remifentanil had an average RHI that was 0.21 lower compared with those not giving remifentanil (CI95 = −0.04 to −0.38, P = 0.02). There was a trend in the same direction for subjects receiving propofol (mean difference −0.16, CI95: −0.02 to −0.35, P = 0.08), and for subjects receiving norepinephrine (mean difference −0.16, CI95: 0.03 to −0.35, P = 0.09). Furthermore, the mixed effect model showed a significant association between RHI and mean arterial pressure: an increase in RHI of 1.0 was associated with an increase in mean arterial pressure of 7 mm Hg (CI95: 2–12 mm Hg, Pmixed-model t-test = 0.01). There were no significant differences in RHI between subjects grouped by coiling and clipping (P = 0.56 for interaction with time; P = 0.14 for main effect).

Average values of plasma arginine:ADMA ratios and serum nitrite/nitrate concentrations are plotted against RHI in Figure 2. There was a significant correlation between subject averaged arginine:ADMA ratios and RHI (r Pearson = 0.43, CI95: 0.15–0.64, P = 0.004), but not between nitrite/nitrate and RHI (r Pearson = −0.07, CI95: −0.36 to 0.23, P = 0.65). In linear
mixed models of all measured values, RHI increased by 0.2 for every increase of 100 of the arginine:ADMA ratio (CI95: 0.1–0.3, \(P_{\text{mixed model} t\text{-test}} = 0.006\)) independent of day after SAH. In a similar model, nitrite/nitrate (log2-transformed) was not significantly associated with RHI (CI95: −0.13 to 0.03, \(P_{\text{mixed model} t\text{-test}} = 0.21\)).
Figure 3 presents serial data of log-transformed S-100B concentrations in relation to 30 day survival, delayed neurological deficit, TCD-vasospasm, and angiographic vasospasm. In the mixed model, there was a significant interaction term between plasma levels of S-100B in relation to day after SAH and survival ($P_{F-test}=0.008$). In non-survivors compared with survivors, values were 2.7 times higher on days 6–8 (CI95: 1.3–5.7, $P_{mixed \ model \ t-test}=0.007$), 1.8 times higher days 9–11 (CI95: 0.9–1.8, $P_{mixed \ model \ t-test}=0.10$), and 4.0 times higher days 12–15 (CI95: 1.9–8.3, $P_{mixed \ model \ t-test}=0.0002$). Subjects surviving to day 30 had gradually decreasing S-100B values reaching 60% of baseline at days 13–15 (CI95: 0.5–0.9, $P_{mixed \ model \ t-test}=0.004$). Subjects with angiographic vasospasm had lower S-100B on days 3–5 and 6–8 by 0.5 (CI95: 0.3–0.9, $P_{mixed \ model \ t-test}=0.03$). Subjects with delayed neurological deficit had higher S-100B than those without from days 6 to 8 and onwards. However, the ratio, 2.1, was only significantly different on days 12–15 (CI95: 1.2–3.8, $P_{mixed \ model \ t-test}=0.01$). RHI was not associated with S-100B at any time after SAH.

**Discussion**

The main finding of this prospective observational study was that flow-mediated vasodilation in peripheral vessels is attenuated in the acute phase after SAH, suggesting that peripheral endothelial dysfunction is part of SAH pathophysiology. RHI measured by fingertip arterial tonometry correlated well...
with arginine:ADMA ratio but not with circulating levels of nitrite/nitrate or with well-known indices of disease severity after SAH, that is, plasma S-100B and $V_{\text{MCA}}$.

We used a non-invasive EndoPat 2000 device for repeated evaluation of peripheral endothelial dysfunction expressed as an RHI value. The method has good agreement with measurements of flow-mediated vasodilation in the brachial artery. Studies in surgical patients and patients with cardiac failure have demonstrated that assessment of peripheral endothelial function by RHI is useful for prediction of cardiovascular adverse effects.

To our knowledge, the current study is the first to report measurements with fingertip arterial tonometry after SAH. Endothelial dysfunction has been frequently observed after ischaemic stroke. In a recent study, Scherbakov and colleagues demonstrated impaired RHI response in the first 5 days after stroke in the territory of the middle cerebral artery. In line with this, we found that endothelial function is impaired in the first days after SAH. The consecutive measurements in the current study, however, showed that RHI, on average, improved thereafter. Notably, in patients who deceased before day 30, RHI did not improve and values tended to be lower compared with survivors. In addition, there was an association between low RHI in the second week after aneurysm rupture and mortality. The significance of these findings has to be tested in larger studies.

ADMA, an endogenous inhibitor of nitric oxide synthases, is a marker of vascular dysfunction and increased values have been associated with mortality in cardiovascular disease. Circulating levels of ADMA have been found to increase after SAH.

**Figure 3** Serial measurements of S-100B in relation to (a) mortality, (b) delayed neurological deficit, (c) TCD vasospasm, and (d) angiographic vasospasm. Points are mean values with 95% confidence limits.
Recently, we reported that low values of the arginine:ADMA ratio are associated with mortality in SAH patients.\textsuperscript{12} The current study extends these data by demonstrating a positive correlation between values of RHI and the arginine:ADMA ratio. This was also observed in stroke patients in whom a diminished arginine:ADMA ratio was associated with reduced RHI.\textsuperscript{26} Thus, a similar mechanism could be active after aneurysmal SAH by which detrimental effects of ADMA contribute to impairment of endothelial function.

The present data suggest that RHI after SAH is neither associated with TCD measurements of $V_{MCA}$ nor plasma levels of nitrite/nitrate. Although the use of TCD is hampered by large measurement variation, it still predicts mortality after SAH.\textsuperscript{11} $V_{MCA}$ in SAH subjects is inversely correlated to plasma levels of nitrite/nitrate and to the 894G/G genotype of the NOS3 gene, but not to arginine, ADMA, or arginine:ADMA ratio.\textsuperscript{12}\textsuperscript{30} $V_{MCA}$ might be primarily governed by the nitrite/nitrate pathway, whereas changes on a microvascular level, as reflected by RHI, are more reliant on the arginine/ADMA pathway.\textsuperscript{12} Further studies are needed to evaluate the RHI response to arginine and nitric oxide donors in aneurysmal SAH.

Plasma concentration of S-100B is associated with outcome after SAH.\textsuperscript{33} Consistent with this, we found that S-100B was significantly higher in non-survivors and in subjects with neurologic deficit. At the same time, S-100B decreased successively in subjects surviving to day 30. Thus, the well-known association of circulating levels of S-100B with cerebral vasospasm and outcome was fully reproduced in this study. The lack of any association between values of RHI and S-100B, and of a significant association between RHI and clinical variables as well, does not favour the use of RHI as an overall outcome marker in aneurysmal SAH.

There are several limitations to the present study. First, the sample size in this single-centre study is too low to allow analysis of the predictive power of PAT measurements. Moreover, given the sample size and number of events, adjustments for further confounding factors are not possible. Secondly, a positive control group with the same incidences of smoking, alcohol abuse, and arterial hypertension as those present in the SAH population was not included. Thus, the degree of endothelial dysfunction before aneurysmal rupture remains unknown. Thirdly, in this observational study, no causal mechanism for the association of arginine/ADMA and RHI can be determined.

In conclusion, we found that endothelial function measured by PAT is impaired in the first days after SAH. Correlation of RHI with the arginine:ADMA ratio suggests that endothelial dysfunction after SAH is coupled to imbalance of the arginine/ADMA pathway. PAT is a feasible technique in patients with SAH, and is relevant to study further in relation to therapeutic intervention and outcome.

**Authors’ contributions**

A.B.: study design, patient recruitment, data collection, data analysis, and writing of the first draft of the paper; J.M.S.: patient recruitment, data collection, and data analysis; B.R.: study design, patient recruitment, and preparation of the paper; N.V.O.: study design and preparation of the paper.

**Acknowledgements**

The authors thank biotechnician Birgit Hein Hansen for skilful laboratory assistance. Analysis kits for S-100B was kindly donated by Roche Diagnostics A/S, Denmark.

**Declaration of interest**

None declared.

**Funding**

This work was supported by the Lundbeck Foundation, Copenhagen, Denmark.

**References**

5. Patvordhan EA, Heffernan KS, Ruan JM, Soffler MI, Karas RH, Kuvin JT. Assessment of vascular endothelial function with peripheral arterial tonometry. *Cardiol Rev* 2010; 18: 20 – 8
24 Stevenson SF, Doubal FN, Shuler K, Wardlaw JM. A systematic review of dynamic cerebral and peripheral endothelial function in lacunar stroke versus controls. Stroke 2010; 41: 434–42

Handling editor: H. C. Hemnings