Low Plasma Arginine:Asymmetric Dimethyl Arginine Ratios Predict Mortality After Intracranial Aneurysm Rupture

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Background—Asymmetrical dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide syntheses, predicts mortality in cardiovascular disease and has been linked to cerebral vasospasm after aneurysmal subarachnoid hemorrhage (SAH). In this prospective study, we assessed whether circulating ADMA, arginine:ADMA ratio, and nitrite/nitrate levels were associated with survival and cerebral vasospasm in SAH patients.

Methods—One hundred and eleven patients were observed day 1 to 15 after SAH, with serial measurements of transcranial Doppler flow velocities ($V_{MCA}$) and plasma biomarkers. Clinical status was assessed by the World Federation of Neurosurgical Societies grading scale.

Results—Overall 30-day mortality was 18%, but differed between patients grouped by low, midrange, and high arginine:ADMA ratio in the first week after SAH. Mortality rates were 14/37, 1/37, and 5/37 in the 3 groups, respectively ($P$-logrank=0.0003). Cox regression showed that low versus midrange or high arginine:ADMA was associated with a hazard ratio of 4.1 independent of World Federation of Neurosurgical Societies grade (95% confidence interval, 1.5–10.9; $P$=0.006). ADMA or arginine:ADMA had no association to $V_{MCA}$, but there was an inverse relationship between $V_{MCA}$ and nitrite/nitrate levels ($P<0.0001$). The $NOS3$ 894G/G genotype was associated with 15% lower $V_{MCA}$ ($P=0.01$). ATbG-$NOS3$ haplotype homozygosity was associated with up to 64% higher nitrite/nitrate levels ($P=0.003$).

Conclusions—This study suggests that plasma arginine:ADMA ratios predict mortality after SAH. Both clinical and physiological measures of changes in cerebral hemodynamics are coupled to the nitric oxide system. (Stroke. 2013;44:1273-1281.)

Key Words: asymmetric dimethyl arginine ■ brain circulation and metabolism ■ cerebral aneurysm ■ genetic polymorphism ■ nitric oxide ■ subarachnoid hemorrhage ■ transcranial Doppler

Impaired cerebral autoregulation and cerebral vasospasm are important factors in the pathophysiology of secondary ischemic neurological deficits after aneurysmal subarachnoid hemorrhage (SAH).1,2 These phenomena occur days to weeks after the primary insult and may lead to poor outcome and death.3 In addition, serious cardiac events occur in about 8% of patients after SAH and are a significant cause of mortality.4 Cardiovascular risk factors such as smoking, alcohol abuse, and arterial hypertension are also risk factors for SAH.5 As a key component in the regulation of vascular tone, the gaseous free radical nitric oxide (NO) is a natural target of investigation in relation to SAH.6 NO biosynthesis occurs mainly through the arginine pathway, but enzymatic reduction of nitrate and nitrite (NOx) as an alternative route to NO formation is likely to exert an important role during hypoxia.7 Experimental models indicate that dysregulation of brain nitric oxide synthases (NOS) occurs after SAH, but the evidence in human subjects regarding the time course of NOx in brain is ambiguous.5–13 Asymmetrical dimethylarginine (ADMA), an endogenous inhibitor of both endothelial (NOS3), inducible (NOS2), and neuronal (NOS1) NOS, has been proposed as a marker of endothelial dysfunction and is associated with mortality rate in several acute and chronic vascular conditions.14–18 In a recent study, plasma ADMA was found to increase over the first week after SAH.19 In addition, high concentrations of ADMA in cerebrospinal fluid have been found to associate with cerebral vasospasm in humans and in primates with experimentally induced SAH.20,21

The aim of this prospective observational study was to investigate if circulating ADMA, arginine, arginine:ADMA ratio, and NOx are associated with survival after SAH. Second, we aimed to determine if changes in the plasma concentrations of these metabolites are related to the World Federation of Neurosurgical Societies (WFNS) clinical grade and transcranial Doppler (TCD) measurements of middle cerebral artery mean flow velocity ($V_{MCA}$). Third, we explored the potential...
impact of common genetic polymorphisms within the dimethylarginine dimethylaminohydrolase gene (DDAH2) and NOS3 on circulating metabolites and V\textsubscript{MCA}.

**Methods**

**Patients**

One hundred and eleven SAH patients were recruited at the Neuromedical Care Unit, Copenhagen University Hospital (Righospitalen) between 2008 and 2012. Ninety-eight were of Northern European origin, whereas 13 were of Inuit (Greenland), Southern European, or Asian ethnicity. Patients were included as early as possible without regard to clinical status, but for logistical reasons, not all patients admitted to the department during the study period were included. The study was approved by the Regional Ethics Board (H-A-2008–033 and H-3-2010-136) and written informed consent was obtained from all participants or next of kin. Part of the data pertaining to the TCD measurements have previously been published in a method validation study.\textsuperscript{22} All subjects had computerized tomography-verified SAH rated by the Fisher scale.\textsuperscript{23} The aneurysmal pathogenesis was diagnosed either during surgery or by computerized tomography angiography or digital subtraction angiography. The aneurysm(s) was secured either by intravascular coiling technique or by surgical clipping, except in 4 cases where a combination or conservative approach was selected (Table 1). Later, adjunctive therapies and computerized tomography scans were administered at the discretion of the treating team of neuroanesthesiologists and neurosurgeons in adherence to the department guidelines, which include oral nimodipine (60 mg/d) for prevention of cerebral vasospasm.

**Clinical Variables**

Patients were included at day 0 to 2 after SAH. Day 0 was defined as the date of the stroke. Observations were taken up to day 15 and a median of 6 observation sets were taken per patient (interquartile range, 3–8). A medical history, including smoking (ever/never), arterial hypertension (deserving medical treatment), and alcohol abuse (ever/never), was obtained as accurately as possible from either the patient or relatives. At each observation, the clinical presentation of the patient was assessed by use of the WFNS grading scale.\textsuperscript{24} WFNS grading was made in conjunction with blood sampling and color-coded TCD (Micromaxx, Sonosite Ltd, Hitchin, UK; P17/5 1MHz probe) of middle cerebral artery VMCA. All observations were made in the interval of 9:00 AM to 12:00 AM. Each TCD session consisted of at least 2 measurements on each side, with at least 5 minutes in between. Survival status was assessed 3 months after inclusion of the last patient.

**High-Performance Liquid Chromatography Analysis of ADMA and L-arginine**

Blood samples were drawn from arterial or venous catheters, and the plasma and serum fractions stored at −80°C. Samples under went solid-phase extraction (Oasis MCX column, Waters) followed by high-performance liquid chromatography separation on a symmetry C18 column, 3.9×15 mm, 5 µm pore (Waters, WAT046980), according to conditions previously described,\textsuperscript{25} with the exception that monoethyl arginine was used as the internal standard at a concentration of 2 µmol/L, as suggested elsewhere.\textsuperscript{26} The high-performance liquid chromatography equipment consisted of a Waters 717plus autosampler, 2 Shimadzu LC-20AD Prominence Pumps, a Shimadzu DGU-20A5 Prominence Degasser, and a Shimadzu RF-20A Prominence Fluorescence Detector. Data collection, peak identification, and calculation of area under the curve were performed with Empower 2.0 software (Waters). Standards were purchased from Sigma-Aldrich except for monoethyl arginine (Enzo Life Sciences).

**NOx Detection**

Serum samples were spun through a 30-kD micropore filter (Nanosep 30k Omega, Pall Corp, Ann Arbor, MI) before duplicate analysis with a commercially available NOx detection kit based on the Griess reaction (cat. 780001, Nitrite/Nitrate Colorimetric Assay Kit, Cayman Chemicals, Ann Arbor, MI).

**Genotyping and Haplotype Estimation**

NOS3 polymorphisms were selected because of previous associations to SAH.\textsuperscript{7–20} The DDAH2 single nucleotide polymorphisms (SNPs) have been shown to be functional in affecting gene expression,\textsuperscript{26} and a pairwise tagging approach (Haplview 4.2) showed >95% coverage (r²=0.8). Genotype analysis was carried out within the subset of patients of Northern European origin (n=98). DNA was extracted from whole-blood samples using the FlexiGene DNA kit (Qiagen, Hilden, Germany, cat.no 12106). In DDAH2, the following SNPs were genotyped: rs805305 (–499G>C), rs9267551 (–1020G>C), and rs2272592 (–1415G>A). In NOS3, the rs1800779 (–922A>G), rs2070744 (–786T>C), and rs1799983 (894G>T, gln298asp) were determined. All SNPs were analyzed with TaqMan assays on an Applied Biosystem 7500 Fast Real-Time PCR device (Applied Biosystems, Lincoln, CA). Genotyping of the intron 4 27-bp variable number of tandem repeats polymorphism followed a previously described PCR/gel electrophoresis protocol (forward prim: 5′-AGG CCC TAT GGT AGT GCC TTG-3′, reverse prim: 5′-TCT CTT AGT GCT GTG AC-3′; MWG-Biotech AG).\textsuperscript{31} Haplotypes were estimated with the software PHASE 2.1 together with a larger group (n=333) of samples from SAH patients that we have at our disposal, to improve accuracy of the estimates.

**Statistical Analysis**

All statistical procedures were carried out with R statistical package 2.15.1.\textsuperscript{32} Graphing was done using the R package ggplot2.\textsuperscript{33} Statistical significance was accepted at the P<5% level.

Prediction of mortality and angiographic vasospasm were tested by Cox regression, and the assumption of proportional hazards was assessed with goodness-of-fit test of Schoenfeld residuals and by visual assessment of the cumulative hazard and log–log survival plots (R library Survival).\textsuperscript{34} After this assessment, it was clear that the proportional hazards could not reasonably be assumed after day 30. We therefore censored survival data at day 30 in the subsequent analysis, and are thus addressing hazards in this time frame. In these models, a subject average of plasma values of arginine, ADMA, arginine:ADMA, and NOx day 1 to 7 (first week) entered the model. To account for possible nonlinear effects on the survival function, we stratified the concentrations in tertiles (low, middle, and high thirds). The minimum WFSN score for each patient during the first week (hereafter WFSN grade) entered the statistical analysis as a surrogate for the combined brain ischemic challenge of stroke, surgery/coiling, and associated complications. The main univariate log-rank tests of arginine, ADMA, arginine:ADMA, and NOx were corrected for multiple comparisons using the false discovery rate method (P\textsubscript{FDR}).\textsuperscript{35} If not explicitly stated uncorrected probability values are presented in the following.

Repeated measures quantitative data were analyzed using linear mixed effects models implemented in the R library nlme, to take into account the unbalanced and within subject correlated nature of the data.\textsuperscript{36} In models of plasma metabolites, subject entered as a random effect (random intercept). Initially, all models included an interaction term between 30-day survival and time (grouped in days 0–5, 6–10, and 11–15), which was removed in case of a nonsignificant term between 30-day survival and time (grouped in days 0–5, 6–10, and 11–15). We therefore censored survival data at day 30 in the subsequent analysis, and are thus addressing hazards in this time frame. In these models, a subject average of plasma values of arginine, ADMA, arginine:ADMA, and NOx day 1 to 7 (first week) entered the model. To account for possible nonlinear effects on the survival function, we stratified the concentrations in tertiles (low, middle, and high thirds). The minimum WFSN score for each patient during the first week (hereafter WFSN grade) entered the statistical analysis as a surrogate for the combined brain ischemic challenge of stroke, surgery/coiling, and associated complications. The main univariate log-rank tests of arginine, ADMA, arginine:ADMA, and NOx were corrected for multiple comparisons using the false discovery rate method (P\textsubscript{FDR}).\textsuperscript{35} If not explicitly stated uncorrected probability values are presented in the following.

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Table 1. Descriptive Population Statistics

<table>
<thead>
<tr>
<th></th>
<th>Absolute</th>
<th>Relative</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>111</td>
<td>1.00</td>
</tr>
<tr>
<td>Sex, female/male</td>
<td>81/30</td>
<td>0.72/0.28</td>
</tr>
<tr>
<td>Acquired risk factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking, ever/never</td>
<td>78/31</td>
<td>0.72/0.28</td>
</tr>
<tr>
<td>Alcohol abuse, ever/never</td>
<td>23/84</td>
<td>0.21/0.79</td>
</tr>
<tr>
<td>Arterial hypertension, yes/no</td>
<td>40/70</td>
<td>0.36/0.64</td>
</tr>
<tr>
<td>Clinical presentation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aneurysm location, ACA/MCA/ICA/VBA</td>
<td>43/27/21/20</td>
<td>0.39/0.24/0.19/0.18</td>
</tr>
<tr>
<td>Surgical/endovascular treatment/other†</td>
<td>40/67/4</td>
<td>0.36/0.60/0.04</td>
</tr>
<tr>
<td>Fisher grade, I/II/III/IV</td>
<td>2/4/87/18</td>
<td>0.02/0.04/0.78/0.16</td>
</tr>
<tr>
<td>Intracerebral hematoma, yes/no</td>
<td>27/84</td>
<td>0.24/0.76</td>
</tr>
<tr>
<td>Intraventricular hemorrhage</td>
<td>67/44</td>
<td>0.60/0.40</td>
</tr>
<tr>
<td>Angiographic vasospasm, yes/no</td>
<td>31/80</td>
<td>0.28/0.72</td>
</tr>
<tr>
<td>NOS3 genotypes (n=98)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−922A&gt;G, AA/AG/GG</td>
<td>41/46/9</td>
<td>0.43/0.48/0.09</td>
</tr>
<tr>
<td>−786T&gt;C, TT/CT/CC</td>
<td>41/46/9</td>
<td>0.43/0.48/0.09</td>
</tr>
<tr>
<td>Intron 4 b/a, bb/ab/aa</td>
<td>73/22/2</td>
<td>0.75/0.23/0.02</td>
</tr>
<tr>
<td>894G&gt;T, GG/GT/TT</td>
<td>41/51/4</td>
<td>0.43/0.53/0.04</td>
</tr>
<tr>
<td>DDAH2 genotypes (n=98)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>−449G&gt;C, CC/CG/GG</td>
<td>40/44/13</td>
<td>0.41/0.45/0.13</td>
</tr>
<tr>
<td>−1026G&gt;C, GG/GG/CC</td>
<td>67/23/5</td>
<td>0.74/0.24/0.02</td>
</tr>
<tr>
<td>−1415G&gt;A</td>
<td>71/23/2</td>
<td>0.70/0.23/0.03</td>
</tr>
</tbody>
</table>

ACA indicates anterior cerebral arteries and anterior communicating artery; CT, computed tomography; DDAH2, dimethylarginine dimethylamino hydrolase gene; ICA, internal carotid trunk and bifurcations; MCA, middle cerebral artery; NOS, nitric oxide synthase; and VBA, vertebrobasilar artery complex.
†Conservative (n=3). Combination of surgical and endovascular intervention after complication (n=1). Only genotypes for patients of Northern European origin were studied.

Results

One hundred and eleven patients aged between 22 and 84 years (mean 55 years) were included in the study. Summary statistics of the studied population are presented in Table 1. Twenty patients died before day 30, whereafter the survival curve flattened and only 1 patient died between day 30 and 100. Survival status was ascertained at least 100 days after SAH and the median follow-up time was 380 days. The number of patients in each WFNS group were I, 37 (33%); II, 22 (20%); III, 6 (5%); IV, 24 (22%); and V, 22 (20%). Because only 6 patients were WFNS grade III, we pooled them with grade II in the subsequent analysis.

Survival and Arginine, ADMA, Arginine:ADMA Ratio, and NOx

The average concentrations in the first week of ADMA, arginine, and arginine:ADMA ratios were grouped in low, midrange, and high tertiles. The cutoff points for plasma arginine:ADMA tertiles were low (77–166), midrange (166 to 211), and high (211 to 343). We then plotted Kaplan–Meier survival curves of each metabolite. Log–rank tests showed that neither ADMA (P=0.45; P₉₀=0.61), arginine (P=0.07; P₉₀=0.13), or NOx (P=0.97; P₉₀=0.97) survival curves differed. However, the low, midrange, and high strata of arginine:ADMA ratios had significantly different Kaplan–Meier curves (P=0.0003; P₉₀=0.002) as shown in Figure 1. In a Cox proportional hazards model, the hazard ratio between low and high range arginine:ADMA ratios was 3.1 (95% confidence interval [CI₉₅], 1.1–8.7; P₉₀=0.028), whereas the hazard ratio was 17 between low and midrange groups (CI₉₅, 2.2–129; P₉₀=0.007). The confidence band is extremely wide, as there was only 1 death in the group with midrange arginine:ADMA, and to obtain a more reasonable estimate, we pooled the mid- and high-range groups. Thus, the hazard ratio for being in the group of low-range arginine:ADMA ratios versus mid- or high-range was 5.4 (CI₉₅, 2.1–14; P₉₀=0.0005). Thus, our data suggest low arginine:ADMA ratio to be a univariate predictor of mortality of clinically relevant magnitude.

We then continued to analyze other potential predictors of mortality. The only significant univariate predictor besides arginine:ADMA ratio was WFNS grade (Figure 1B; P₉₀=0.0007; P₉₀=0.003; grade I versus V: hazard ratio [HR], 7; CI₉₅, 2.2–26; P₉₀=0.002). Notably, there were no statistically significant differences in the survival curves of WFNS grades I to IV (Figure 1B). Arterial hypertension was a borderline significant predictor, but did not stand for multiple testing correction (P₉₀=0.02; P₉₀=0.053; HR, 2.8; CI₉₅, 1.2–6.9). Age, smoking, aneurysm site, Fisher grade, or angiographic vasospasm were not associated with survival rate before probability value adjustment.

We suspected the effect of low arginine:ADMA on mortality risk to be mediated simply by the association to the WFNS grade. Thus, we stratified the population by WFNS grades I to IV versus grade V, pooling grades I to IV because they have similar survival rates (figure 1B). Then, 2-way contingency tables of 30-day survival and arginine:ADMA tertiles of low values versus midrange and high values were constructed (Table 2). In this crude test, the association was significant in the grades I to IV group (n=89; P=0.02), whereas the grade V group was not powered to detect an association (n=22). We then tested for interaction between arginine:ADMA and base WFNS grade in a Cox model. There was no significant interaction (P₉₀=0.85), so the model reduced to additive effects of arginine:ADMA (HR for low versus middle/high, 4.1; CI₉₅, 1.5–10.9; P₉₀=0.006), and WFNS grade (HR for grade V versus I to IV, 3.5; CI₉₅, 1.4–8.7; P₉₀=0.007). In summary, the analysis show that low arginine:ADMA ratios and WFNS grade V independent of another are predictors of mortality after SAH of about equal strength.

Changes in Circulating ADMA, Arginine, and NOx After SAH in Relation to Clinical Grade

In the following, all percentages refer to the coefficient estimates of the mixed model (back-transformation from the log scale). The changes over time in ADMA, arginine, arginine:ADMA, and NOx are shown in Figure 2. Both ADMA and L-arginine concentrations increased in plasma in the first 10 days after SAH. Whereas good grade patients (WFNS I–III) reached a plateau days 11 to 15, poor grade...
patients (WFNS IV and V) continued to augment their values (P for interaction=0.0007 and 0.04 for ADMA and arginine, respectively). Without regard to clinical condition, the overall change in ADMA from day 0 to 5 to day 6 to 10 was 13% (CI95, 9%–16%). There was no change over time in arginine:ADMA ratio (P=0.57), but a consistent highly significant difference between clinical grades (P<0.0001), equaling a 27% lower ratio in patients of WFNS grade V than grade I (CI95, 37%–16%; P<0.0001). NOx changes over time depended significantly on WFNS grade (P=0.0009). Thus, in poor grade patients, the NOx level in the initial 5 days was 45% higher than in grade I (CI95, 5%–102%; P=0.03). On day 11 to 16, the NOx level declined to lower levels in poor grade patients (35%) than grade I (CI95, 7%–54%; P<0.02).

**Middle Cerebral Artery Flow Velocity**

Serum NOx was negatively correlated with VMCA (Figure 3A). Thus, a 10 µmol/L increase in serum NOx corresponded to a decrease in VMCA of 6% (CI95, 3%–9%; P<0.0001). Neither plasma ADMA, arginine, or arginine:ADMA ratio were associated with VMCA. However, a 22% reduction in NOx levels was associated with a doubling of the arginine:ADMA ratio independent of time after SAH (Figure 3B; CI95, 8%–33%; P=0.003). Patients dying before day 30 tended to have lower VMCA (10%) in the initial 5 days (CI95, –23% to +6%; P=0.08). But, in days 5 to 10, these patients had 17% higher velocities than survivors (CI95, 6%–30%; P=0.002).

**Angiographic Vasospasm**

Patients with angiographic vasospasm did not differ significantly in arginine or ADMA levels compared with patients without angiographic vasospasm, but had a 15% higher arginine:ADMA ratio on day 5 to 10 (CI95, 5% to 26%; P=0.003). Serum NOx in the same period was 39% lower (CI95, –23% to –52%; P<0.0001) compared with patients without angiographic vasospasm, and continued to be lower.
Table 2. Arginine:ADMA Ratio and 30-Day Survival Stratified by Clinical Grade

<table>
<thead>
<tr>
<th>Clinical Grade</th>
<th>Arginine:ADMA tertiles</th>
<th>Survival to Day 30</th>
<th>p value</th>
<th>OR†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>Low</td>
<td>Yes: 18 (75%)</td>
<td>6 (25%)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Middle</td>
<td>32 (100%)</td>
<td>0 (0%)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>29 (88%)</td>
<td>4 (12%)</td>
<td>5.1 (1.3–20)</td>
</tr>
<tr>
<td>Low</td>
<td>High</td>
<td>5 (38%)</td>
<td>8 (62%)</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>Middle</td>
<td>4 (80%)</td>
<td>1 (20%)</td>
<td>5.6 (0.81–39)</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>3 (75%)</td>
<td>1 (25%)</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Note: p value < 0.05. OR†, odds ratio with 95% confidence limits.

Day 11 to 15 (−29%; CI95, –7% to –46%). Neither of the studied polymorphisms had any association with angiographic vasospasm. In particular, for the −786T>C SNP, which has previously been associated with vasospasm, there was 6 versus 4 C/C, 37 versus 14 C/G, and 32 versus 12 G/G in patients without and with angiographic vasospasm, respectively (P = 0.67).

Genetic Polymorphisms and Haplotypes Within DDAH2 and NOS3 and Intermediary Metabolites

Genotype distributions within the 98 SAH patients of Northern European origin are presented in Table 1. All polymorphisms were within Hardy Weinberg equilibrium except the 894G>T (Exact test: P = 0.02; Pfdr = 0.13). None of the DDAH2 polymorphisms or haplotypes were associated with plasma ADMA or arginine:ADMA levels. Three haplotypes had a frequency >5%, namely −445G/−1020G/−1415G (45%), −445C/−1020G/−1415G (36%), and −445G/−1020C/−1415A (13%).

In all 4 NOS3 variants, there was a trend toward major allele homozygotes having generally lower NOx levels than minor allele carriers. Thus, in mixed effects ANOVA corrected for time after SAH, the −922A/A genotype had 30% higher NOx levels (CI95, 4%–62%; P = 0.024), the −786T/T genotype had 19% higher NOx levels (CI95, –0% to 50%; P = 0.125), the intron 4 b/b genotype had 18% higher NOx levels (CI95, −9% to 53%; P = 0.20), and the 894G/G genotype had 29% higher NOx levels (CI95, 3%–61%; P = 0.029). Four NOS3 haplotypes had a frequency >5%, ATbG (52%), ATbT (11%), GCbT (17%), and GCaG (11%). The 24% of the study population with ATbG homozygosity had, on average, 60% higher NOx versus noncarriers (CI95, 14%–124%; P = 0.007). All other haplotypes tended (nonsignificantly) to have lower NOx for both homozygotic and heterozygotic carriers versus noncarriers. As the NOx levels were correlated to VMCA, we proceeded to test if there was an association with NOS3 −922 A/A or 894G/G and VMCA, whereas adjusting for NOx. The 894G/G genotype was associated with 15% lower VMCA (CI95, 5%–25%; P = 0.012) compared with G/T and T/T genotypes. There was no association with the −922A/A and VMCA (P = 0.81). Homozygosity for the ATbG haplotype was not correlated with VMCA (CI95, –15% to +9%; P = 0.28).

Discussion

Key Findings

This study shows that a low arginine:ADMA ratio in the first week after SAH predicts mortality rate in the first 30 days (Figure 1). The hazard ratio was comparable with that of the most severe clinical grade (WFNS V), and the association was independent in a Cox model adjusted for clinical grade (HR, 4.1; P = 0.006). Second, we found the arginine:ADMA ratio to be negatively correlated with the serum NOx level (Figure 3B), and that decreasing NOx levels were associated with higher VMCA (Figure 3A). Furthermore, we report an association between the NOS3 894G/G genotype and about 29% higher overall NOx levels (Figure 3B). The effect was found to be specific to the ATbG-haplotype, which had 60% higher NOx levels. Finally, the NOS3 894G/G genotype, but not haplotypes, associated with a 15% lower VMCA independent of NOx (Figure 3A). We also described the temporal development in circulating arginine, ADMA, and NOx, which involves steadily climbing ADMA and arginine values in the first 10 days after SAH, whereas WFNS grade I to IV patients reached a plateau, whereas WFNS grade I to IV patients continued to climb. The arginine:ADMA ratio was higher in good grade patients, but did not change significantly over time. NOx levels were initially higher in grade V patients, but then declined to levels below better grade patients in day 11 to 15. Altogether, these results suggest that peripheral metabolites within the NO pathway are associated to the pathophysiology in the subacute phase after SAH, affecting the mortality rate possibly through modulation of cerebral blood flow.

Mortality

ADMA infusion in healthy subjects reduces cerebral blood flow and cardiac output. Epidemiological studies have identified ADMA as a predictor of ischemic brain lesions and endothelial dysfunction. Low plasma arginine:ADMA ratio (1st quartile) was an independent predictor of mortality in a cohort of patients with dilated cardiomyopathy. High ADMA has been associated with increased mortality in patients with unstable angina and coronary artery disease, chronic heart failure, and peripheral artery disease. Notably, in these studies, the association seems to rest solely with the extreme quartile or tertile, which is similar to our findings, and may indicate the presence of a critical threshold. With the relatively low number of events in our sample, testing for independence in multivariate models is not feasible. However, even in analysis stratified by the only other univariate predictor, base WFNS grade, the association was still significant. This signifies that in WFNS grade I to IV patients, in which the exact grade is less specific to mortality risk, low...
plasma arginine:ADMA ratio is still a predictive marker of mortality risk.

Time Course

Rodling-Wahlström and colleagues reported an increase in plasma ADMA of about 68% within the first week after SAH, but did not measure arginine. ADMA levels were within the 0.2 to 0.4 μmol/L range, which is similar to our findings, though we did not see such a marked increase. Peripheral endothelial dysfunction and reduced plasma L-arginine:ADMA ratio have been shown to occur in the first 5 days after ischemic stroke, which is different from our observation: that whereas changes in ADMA and arginine do occur after SAH, the arginine:ADMA ratio remains overall stable.

Relation Between Middle Cerebral Artery Blood Flow Velocity, NOx, and arginine:ADMA

TCD is a modality associated with high measurement variation and correlates with both reduced large-vessel diameter and increased blood flow, which makes interpretation difficult. Furthermore, the clinical value of TCD has been questioned. Our finding that neither ADMA, arginine, or arginine:ADMA was related to VMCA is in line with previous findings, though they are limited in numbers. Biological explanations may be that first, systemic ADMA is not a reflection of intracellular concentrations; second, the plasma ADMA is too low in concentration to limit arginine transport; and third, the effect of ADMA may be on the microvascular level in reducing blood flow. In contrast to this, we found that higher serum NOx concentration associated with lower VMCA (Figure 3A). This
could indicate that blood flow in cerebral conductance vessels is reliant on the NOx pathway more than the arginine pathway in NO biosynthesis after SAH. This is meaningful in that this pathway is relatively more important during hypoxia. In support of this, studies show that in monkeys with experimental SAH, infusion of sodium nitrite ameliorates middle cerebral artery constriction, whereas arginine infusion does not have this effect, though it still increases cerebral blood flow. Also, nitrite infusion increased cerebral blood flow in rats during NG-nitro-L-arginine methyl ester inhibition of NOS.

Role of Genetic Variation in DDAH2 and NOS3

Our findings indicate that common genetic variations in the promoter region of DDAH2 are not associated with plasma ADMA. With respect to the NOS3 gene, we observed that all major allele homozygotes, within the 4 studied polymorphisms, tended to have higher circulating NOx levels, and that this effect seems to be mediated by an association to the predominant haplotype, ATbG. Furthermore, we found that the 894G/G genotype was associated with lower $V_{MCA}$ independent of the association to NOx, whereas none of the other polymorphisms or the ATbG haplotype were correlated this way. We regard these as interesting hypothesis generating findings that deserves further studying, especially given the conflicting evidence regarding the –786T/C SNP and an association to symptomatic or angiographic vasospasm, which we did not find either. However, follow-up angiography was only done on clinical indication (not systematically), which may have biased this result.

Limitations

The current study was conducted as a single-center study, and our finding that plasma arginine:ADMA ratio is associated with mortality risk after SAH warrants replication in a larger cohort, with the ability to adjust for further confounding factors than we were able to in the present material. We did not include all patients admitted to the hospital in the study period because of practical limitations. This may unknowingly have introduced some selection bias into our sample, meaning that the external validity may have suffered. We did not blind investigators, but samples were anonymized and measurements were performed without knowledge of outcome variables. Also, given the sample size and number of events, we cannot sensibly adjust for many confounding variables. Thus, we could potentially have overlooked an explanatory factor that both affects mortality and the plasma arginine:ADMA ratio.

Implications of This Study

An ideal biomarker would allow timely prognostication of patients, regardless of clinical grade.

Our study suggests that whereas changes in arginine and ADMA occur after SAH, the arginine:ADMA ratio is stable over time and correlated to clinical grade. This raises the question if interventions to normalize the arginine:ADMA ratio can improve endothelial function and patient outcome, or if this biomarker is simply a sign of vascular damage already done. The idea of specific pharmacological modulation of endothelial function is tempting, especially because it seems that blocking whole pathways within this system might ameliorate certain clinical signs in one organ system, whereas damaging the other. Potential modulators include statins, certain angiotensin converting enzyme inhibitors, sodium nitrite, or isoform-specific NOS inhibitors.

In conclusion, this study suggests that an early low plasma arginine:ADMA ratio within the first week after SAH is predictive of poorer survival rate. The association was independent of clinical WFNS grade, although this conclusion relies on low patient numbers. We reported on the temporal changes in ADMA, arginine, arginine:ADMA ratio, and NOx and found an association between $V_{MCA}$ and serum NOx, but none with arginine or ADMA. Also, homozygosity of the NOS3 –922A/–786T/intr4b/894G haplotype was associated with...
60% higher NOx levels. Altogether, our findings add to the growing evidence that endothelial dysfunction and imbalance in the NO system are essential factors in the pathophysiology of SAH in humans.

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Disclosures

None.

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Low Plasma Arginine:Asymmetric Dimethyl Arginine Ratios Predict Mortality After Intracranial Aneurysm Rupture
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arginine:ADMA ratio (average of week 1)

Survival status day 30
- died
- survived

WFNS grade
- I
- II/III
- IV
- V
N = 111. Points show patient average arginine:ADMA ratio of week 1 measurements. Points are jittered horizontally to avoid overplotting. Dotted lines indicate 1st and 2nd tertile (which formed the basis of the presented survival analysis). Boxplots in grey show median, 1st and 3rd quartile of base WFNS groups.