Aim: Increased levels of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase, have been observed in patients with cardiovascular risk factors and atherosclerosis and in patients with a history of stroke. The role of ADMA and its analogue symmetric dimethylarginine (SDMA) in acute ischemic stroke is yet unclear. We hypothesized that plasma dimethylarginine levels increase in the hyper-acute phase after ischemic stroke and that their time course is related to stroke outcome.

Methods: Plasma dimethylarginines ADMA and SDMA and L-arginine levels were measured in 67 patients at 6, 12, 24 hours, as well as 3 and 7 days after stroke onset using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS-MS). Data were compared to control data from 32 age-adjusted healthy volunteers. Clinical outcome was assessed using the modified Rankin Scale (mRS) at 90 days after stroke.

Results: At baseline, plasma ADMA levels were higher in stroke patients than in controls, whereas plasma SDMA and L-arginine levels did not differ from control subjects. The time courses of ADMA and SDMA were related to the clinical outcome. Binary logistic regression analysis showed that ADMA levels of $\geq 0.566 \text{ mol/L}$ at day 3, $\geq 0.530 \text{ mol/L}$ at day 7 and SDMA levels of $\geq 0.59 \text{ mol/L}$ at 24 hours predicted an unfavorable clinical outcome.

Conclusions: An increase of both ADMA and SDMA plasma levels within the first 72 hours after the onset of ischemic stroke predicts a poor outcome.


Key words: Asymmetric dimethylarginine (ADMA), Symmetric dimethylarginine (SDMA), L-arginine, Stroke, Outcome
and in subjects with a history of stroke. Currently, ADMA is considered to be not only a marker but also a mediator of oxidative stress via the inhibition and uncoupling of NOS. Oxidative stress induces the expression of inflammatory genes and thereby enhances atherosclerotic processes.

Recently, ADMA levels have been shown to be decreased in patients in the chronic stage after ischemic stroke at least 3 months after the event when treated with statins in comparison to the non-statin-treated group. In another study, treatment with statins resulted in a significant reduction of markers of oxidative stress.

The structural isomer of ADMA, SDMA, has no effect on nitric oxide synthesis but is also related to cardiovascular events. SDMA has also been shown to be an independent predictor of overall mortality after acute stroke. To the best of our knowledge there has been no report on the relationship between dimethylarginine, i.e., ADMA and SDMA plasma levels during the hyper-acute phase of ischemic stroke, and functional outcome. Dimethylarginine data for the first hours after stroke onset are available only from a study where ADMA levels in the cerebrospinal fluid (CSF), not in the plasma, were directly related to stroke severity on admission and stroke outcome at 90 days after stroke.

In the present study we serially analyzed the dimethylarginine plasma concentration after acute ischemic stroke to elucidate its release over time and its possible relation to stroke outcome. We also investigated L-arginine, the substrate for nitric oxide synthase.

**Materials and Methods**

**Study Population**

Between January 2007 and January 2009, 67 patients with ischemic stroke were prospectively included in this study within 6 hours of symptom onset. Ischemic stroke was defined as an acute focal neurological deficit with cranial computed tomography (CCT) or magnetic resonance imaging (MRI) evidence of infarction. Exclusion criteria were transient ischemic attack (TIA), hemorrhagic stroke, history of malignant tumor, functional deficit prior to the index event and severe systemic inflammatory disease with a serum C-reactive protein level > 50 mg/L and/or need for intravenous antibiotic treatment.

Stroke severity on admission was assessed by the National Institute of Health Stroke Scale Score (NIHSS). NIHSS 0-1 was considered as mild stroke, 2-8 as moderate stroke and ≥9 as severe stroke. The primary outcome was assessed by the modified Rankin scale (mRS) at 90 days after stroke. mRS 0-1 at 90 days after stroke was considered a favorable outcome, and mRS 2-6 an unfavorable outcome.

Clinical data recorded from all patients included demographic characteristics, baseline stroke severity (NIHSS on admission), stroke etiology classified according to TOAST criteria, stroke risk factors, such as hypertension, diabetes mellitus, hyperlipidemia and smoking status, and creatinine on admission. Nine patients had a history of stroke. In each case, the event had occurred at least 3 months previously. Two of these patients had residual symptoms without functional disability; one had unilateral sensory disturbance of the upper extremities, and the other had slight unilateral facial paresis.

Between April 2010 and July 2010, 32 healthy volunteers without malignant tumor and without an event of stroke or myocardial infarction at least during the previous two years provided blood samples for comparison with the patient data. Most were relatives of stroke patients.

The study was approved by our local ethics committee, and patients and volunteers gave informed consent.

**Blood Collection**

Serum and plasma samples were drawn from the patients 6h, 12h, 24h, 3d and 7d after stroke onset and stored at −80°C until analysis.

In 34 patients, blood was collected within six hours after stroke onset (“2-6-hour samples”). Five patients were lost to follow-up over the first week, leading to 64 blood samples at 3 days and 62 samples at 7 days. In 55 patients, blood samples were also collected 90 days after stroke.

**Measurement of ADMA, SDMA, L-arginine and Other Markers**

Plasma ADMA, SDMA and L-arginine levels were assessed using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS-MS). The intra-day precision was 4.6% for ADMA, 6.1% for SDMA, and 4.3% for L-arginine. The inter-day precision was 4.6% for ADMA, 5.2% for SDMA, and 4.5% for L-arginine. The intra-day precision data were generated by measuring plasma quality control samples in one batch (n=10), whereas the inter-day precision data were derived from measurements of plasma quality control samples taken on different days (n=6). Creatinine and hsCRP were measured using certified laboratory tests in the Department of Clinical Chemistry at Hannover Medical School, Germany.
Statistical Analyses

Statistical analysis was performed with the SPSS software package version 11.5. Data are presented as a portion for categorical variables and as the median with interquartile range for continuous variables. The data were tested for statistically significant differences between groups by one-way ANOVA for continuous data and Pearson’s Chi-square test or Fisher’s exact test for categorical data. Differences between patient levels of ADMA, SDMA and L-arginine and levels in the control group were calculated by Student’s t test. Within-group comparisons of the biomarkers at different time points were analyzed by repeated measures ANOVA.

Cut-off points of dimethylarginines and L-arginine were determined using the receiver operating characteristics (ROC) curve and Youden index.

The influence of ADMA, SDMA and L-arginine levels on functional outcome was assessed using binary logistic regression after adjusting for main baseline variables related to outcome in univariate analyses (enter approach and probability of entry p<0.05). Results are presented as adjusted odds ratios (OR) with the corresponding 95% confidence intervals (95% CI). A p value <0.05 was considered significant.

Results

Baseline clinical characteristics, vascular risk factors, stroke type, stroke severity and renal function at baseline are shown in Table 1.

Dimethylarginine Time Courses After Acute Ischemic Stroke

The dimethylarginine levels in the control group were 0.447 (range: 0.349-0.606) µmol/L for ADMA, 0.546 (range: 0.473-0.630) µmol/L for SDMA, and 74.7 (range: 40.7-104.4) µmol/L for L-arginine. ADMA levels were significantly higher in the stroke patient group at any time point compared to controls while SDMA and L-arginine were not (Table 2). ADMA (p=0.008, F=3.858) and SDMA (p=0.008, F=3.868) levels increased significantly during the first 3 days after stroke onset, while L-arginine levels (p=0.247, F=1.400) tended to decrease (Fig. 1).

Forty-one of the 67 patients had a favorable outcome 90 days after stroke, but 26 patients had an unfavorable outcome. In patients with a favorable outcome, ADMA increased during the first 12 hours after stroke onset and then remained stable until day 3 before it decreased again. In patients with an unfavorable outcome, the ADMA level increased until day 3, remained at that level until day 7 and had decreased

Table 1. Baseline Clinical Characteristics of Patients and Healthy Controls

<table>
<thead>
<tr>
<th></th>
<th>Favorable outcome (n = 41)</th>
<th>Unfavorable outcome (n = 26)</th>
<th>Controls (n = 32)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>69 (62, 74)</td>
<td>75.5 (58, 85)</td>
<td>71 (61, 76)</td>
<td>0.177</td>
</tr>
<tr>
<td>Gender (female), %</td>
<td>31.7</td>
<td>65.4</td>
<td>56.3</td>
<td>0.016</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>58.5</td>
<td>65.4</td>
<td>75</td>
<td>0.340</td>
</tr>
<tr>
<td>Hyperlipidemia, %</td>
<td>43.9</td>
<td>42.3</td>
<td>53.1</td>
<td>0.668</td>
</tr>
<tr>
<td>Diabetes, %</td>
<td>7.3</td>
<td>7.7</td>
<td>12.5</td>
<td>0.751</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>19.5</td>
<td>11.5</td>
<td>18.8</td>
<td>0.708</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.76 (24.8, 29.32)</td>
<td>25.1 (23.04, 27.76)</td>
<td>25.59 (23.92, 29.38)</td>
<td>0.359</td>
</tr>
<tr>
<td>Stroke severity on admission, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild</td>
<td>26.8</td>
<td>11.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>65.9</td>
<td>34.6</td>
<td></td>
<td>0.000</td>
</tr>
<tr>
<td>Severe</td>
<td>7.3</td>
<td>53.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stroke etiology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atherothrombotic, %</td>
<td>22.0</td>
<td>11.5</td>
<td></td>
<td>0.566</td>
</tr>
<tr>
<td>Cardioembolic, %</td>
<td>31.7</td>
<td>38.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lacunar, %</td>
<td>36.6</td>
<td>46.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Others, %</td>
<td>9.8</td>
<td>3.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>eGFR, mL/min per 1.73 cm²</td>
<td>80 (64.25, 92.95)</td>
<td>74.1 (46.55, 89.425)</td>
<td>71.3 (61.55, 89.9)</td>
<td>0.219</td>
</tr>
</tbody>
</table>

BMI: body mass index; eGFR: estimated glomerular filtration rate.
The data were tested for statistically significant difference between groups by one-way ANOVA for continuous data and Fisher’s exact test or Pearson’s Chi-square test for categorical data.
Table 2. Levels of Dimethylarginine in patients

<table>
<thead>
<tr>
<th></th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
<th>3d</th>
<th>7d</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADMA</td>
<td>Median</td>
<td>(umol/L)</td>
<td>Median</td>
<td>(umol/L)</td>
<td>Median</td>
</tr>
<tr>
<td>p</td>
<td>0.032</td>
<td>(0.429, 0.548)</td>
<td>0.000</td>
<td>(0.444, 0.586)</td>
<td>0.000</td>
</tr>
<tr>
<td>SDMA</td>
<td>Median</td>
<td>(umol/L)</td>
<td>Median</td>
<td>(umol/L)</td>
<td>Median</td>
</tr>
<tr>
<td>p</td>
<td>0.200</td>
<td>(0.421, 0.619)</td>
<td>0.633</td>
<td>(0.425, 0.641)</td>
<td>0.620</td>
</tr>
<tr>
<td>L-Arg</td>
<td>Median</td>
<td>(umol/L)</td>
<td>Median</td>
<td>(umol/L)</td>
<td>Median</td>
</tr>
<tr>
<td>p</td>
<td>0.884</td>
<td>(56.30, 87.90)</td>
<td>0.254</td>
<td>(51.17, 86.55)</td>
<td>0.079</td>
</tr>
</tbody>
</table>

ADMA: asymmetric dimethylarginine; SDMA: symmetric dimethylarginine; L-Arg: L-arginine
Data are presented as the median and quartile. *P*-values indicate differences between patient levels of ADMA, SDMA and L-arginine and levels in the control group calculated by Student’s *t* test.

Fig. 1. Time courses of dimethylarginine levels after acute ischemic stroke onset (mean ± SD). A: ADMA; B: SDMA; C: L-arginine.
*compared to control, *p* < 0.05, *compared to 6 hours, *p* < 0.05

again 90 days after stroke. At day 3 (*p* = 0.025) and
day 7 (*p* = 0.001), ADMA levels were significantly
higher in patients with an unfavorable outcome than
in those with a favorable outcome (Fig. 2A).

SDMA levels remained unchanged in patients
with a favorable outcome while they continuously
increased over the first 24 hours after stroke onset in
those with unfavorable outcome and then decreased
again. The SDMA level in patients with an unfavor-
able outcome was significantly higher at 12 hours
(*p* = 0.003), 24 hours (*p* = 0.001) and 3 days (*p* = 0.023)
compared to those with a favorable outcome (Fig. 2B).

L-arginine levels did not significantly differ
between the two groups (*p* > 0.05) (Fig. 2C).

Association of ADMA and SDMA with Neurological Outcome in Stroke Patients

ROC analysis was performed for ADMA 3d and
7d and SDMA 12h, 24h and 3d after stroke onset
(Fig. 3). A possible cut-off point for the discrimina-
tion of dichotomized neurological outcome (favorable outcome: mRS 0 or 1 vs. nonfavorable outcome: mRS 2-6) for ADMA at 3d after stroke was calculated as 0.566 μmol/L, and at 7d as 0.530 μmol/L. For SDMA, the cut-off points were 0.519 μmol/L at 12h and 0.591 μmol/L at 24h. We categorized ADMA 3d and 7d, SDMA 12h and 24h into two levels according to the cut-off points determined above. Univariate analysis found that ADMA 3d ≥0.566 μmol/L, ADMA 7d ≥0.530 μmol/L, SDMA 12h ≥0.519 μmol/L and SDMA 24h ≥0.591 μmol/L predicted an unfavorable outcome on 90d after stroke onset. Higher ADMA levels at 3d and 7d and SDMA levels at 24h were independent markers of an unfavorable outcome in multivariate analysis considering all parameters, which were significant in univariate analysis as well as gender and stroke severity on admission (Model 1). The results did not change when age, hypertension and eGFR (estimated glomerular filtration rate) were also considered (Model 2) (Table 3).

**Discussion**

To the best of our knowledge, this is the first study to investigate the time course of plasma dimethyl-arginines and L-arginine after acute ischemic stroke as well as their relation to the clinical outcome at 90 days.

The pertinent findings of our study are: 1) Plasma ADMA levels increased in the hyperacute phase of ischemic stroke irrespective of stroke outcome, SDMA levels increased in patients with an unfavorable outcome, exclusively, while L-arginine levels did not change; 2) the time profile of ADMA and SDMA alterations was related to stroke outcome; 3) high ADMA and SDMA predicted an unfavorable outcome.

**High ADMA in Hyperacute Ischemic Stroke**

ADMA and SDMA originate from the degradation of methylated proteins, which are catalyzed by protein arginine methyltransferases (PRMTs). After proteolysis of the methylated protein, ADMA and
SDMA are released. More than 90% of ADMA is metabolized through DDAHs (dimethylaminohydrolases) while, in contrast, SDMA is completely excreted by the kidney\textsuperscript{21}. Acute ischemic stroke induces oxidative stress in the region of the lesion, which causes an increase in the expression of PRMTs and decreased enzymatic activity of DDAHs\textsuperscript{9, 21}. Both mechanisms - one by increased production, the other by decreased metabolization - may be responsible for the elevation of ADMA and SDMA.

Aside from ADMA, SDMA production/release should also be increased in stroke patients, but SDMA increases may be concealed and SDMA concentrations could have remained quite stable after stroke, at least in patients without severe renal dysfunction due to renal excretion.

**Dimethylarginine Levels and Stroke Outcome**

In this study, stroke outcome was worse in patients with increased ADMA levels than in those with stable ADMA levels. The mechanism behind this is not clear, but it can be hypothesized that increased ADMA levels impair the activity of endothelial NO synthase (eNOS) and thereby inhibit the production of endothelial NO and cerebral perfusion. This hypothesis is supported by several *in vitro* and animal studies showing that ADMA increases vascular tone in cerebral blood vessels\textsuperscript{22, 23}. Moreover, ADMA infusion in healthy volunteers reduced cerebral perfusion significantly\textsuperscript{24}. Furthermore, there is an association of ADMA levels in the CSF and cerebral vasospasm in a primate model of subarachnoid hemorrhage\textsuperscript{25}. Accordingly, ADMA may impair perfusion in the penumbra and might therefore be pathophysiologically relevant.

Fig. 3. ROC curve of ADMA at 3 and 7 days, and SDMA at 12 and 24 hours.
The role of SDMA is less clear than that of ADMA. SDMA has been shown to be a good marker of renal function with high temporal resolution to identify changes in renal function, so the increase in SDMA might be a reflection of (minor) changes in renal function that were not detected by creatinine measurements. This seems possible as even small changes in renal function have been shown to be related to adverse outcomes in neurological emergencies as well as long-term outcome after acute stroke. Aside from being a marker of renal dysfunction, SDMA interferes with the uptake of L-arginine and may thereby (indirectly) inhibit NO production. Brouns et al. reported that higher SDMA levels in the CSF were correlated with a poor outcome at 3 months after stroke onset, but after multivariate analysis this correlation remained only weak. Recently, Schultz et al. reported that SDMA predicted all-cause mortality in stroke patients during 7.4 years of follow-up even after adjustment for renal function. In this study SDMA has also been shown for the first time to affect cerebrovascular mortality. Experimental data suggest that SDMA triggers vascular pathology by modulating store-operated calcium channels in monocytes. It might also be important in modulating the function of monocytes recruited to the site of the ischemic lesion after stroke onset. SDMA may enhance reactive oxygen species (ROS) production in those monocytes, thereby causing blood brain barrier damage and neuronal cell death. Furthermore, inhibited L-arginine uptake by SDMA may induce L-arginine deficiency in neuronal cells. In consequence, neuronal NOS produces huge amounts of superoxide instead of NO, which leads to lipid, protein and DNA damage in the cells and contributes to a poor outcome after stroke.

Despite being the first prospective clinical study in the field, our study has some limitations. Firstly, the number of patients included is rather small considering the multifactorial pathology of ischemic stroke, but we were able to collect serial blood samples from all of our patients, which had never been achieved. Secondly, patients with mild stroke predominated in the present study (NIHSS range 0 to 25, median 4), although the neurological examination was augmented by brain imaging in all of our patients. Thirdly, at the time of blood withdrawal, ischemic stroke patients were not in a fasting state since this conflicted with the strict time course of blood sampling, particularly during the first day. Lastly, the very nature of our work does not allow the elucidation of pathophysiological concepts, i.e. clarify whether elevated ADMA and SDMA just indicate or actually cause an adverse clinical outcome.

In conclusion, our study is hypothesis generating in nature and suggests that both an increase of ADMA known as a marker of cardiovascular risk and atherosclerosis and an increase of SDMA plasma levels after acute ischemic stroke predict poor functional outcome at 90 days after stroke onset. Based on our findings, we believe that the prognostic value and pathophysiological role of ADMA and SDMA in this setting deserve further investigation in larger patient populations.

### Source of Funding

This work was supported by a grant from the HiLF program of Hannover Medical School awarded to Hans Worthmann.

### Table 3. Separate Logistic Regression Models for ADMA (3 and 7 Days) and SDMA (12 and 24 Hours) and Stroke Outcome at 90 Days after Stroke Onset

<table>
<thead>
<tr>
<th></th>
<th>Univariate</th>
<th>Model 1</th>
<th>Model 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ADMA 3 days</strong></td>
<td>4.727 (1.549, 14.427)</td>
<td>7.201 (1.772, 29.263)</td>
<td>6.527 (1.537, 27.723)</td>
</tr>
<tr>
<td></td>
<td>p = 0.007</td>
<td>p = 0.006</td>
<td>p = 0.011</td>
</tr>
<tr>
<td><strong>ADMA 7 days</strong></td>
<td>8.964 (2.660, 20.207)</td>
<td>6.529 (1.802, 23.664)</td>
<td>7.191 (1.734, 29.82)</td>
</tr>
<tr>
<td></td>
<td>p = 0.000</td>
<td>p = 0.004</td>
<td>p = 0.007</td>
</tr>
<tr>
<td><strong>SDMA 12 hours</strong></td>
<td>3.044 (1.073, 8.633)</td>
<td>1.884 (0.571, 6.217)</td>
<td>1.677 (0.475, 5.918)</td>
</tr>
<tr>
<td></td>
<td>p = 0.045</td>
<td>p = 0.298</td>
<td>p = 0.281</td>
</tr>
<tr>
<td><strong>SDMA 24 hours</strong></td>
<td>5.689 (1.928, 16.788)</td>
<td>5.177 (1.508, 17.769)</td>
<td>7.159 (1.670, 30.693)</td>
</tr>
<tr>
<td></td>
<td>p = 0.002</td>
<td>p = 0.009</td>
<td>p = 0.008</td>
</tr>
</tbody>
</table>

Covariant factors included in model 1: gender and stroke severity on admission.
Covariant factors included in model 2: age, gender, stroke severity on admission, hypertension and CKD-EPI-GFR.
Disclosures

Dr. Kielstein runs and hosts the website www.adma.com.
Shufens Chen was funded by a grant from Boehringer-Ingelheim, Germany.

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