Asymmetric Dimethylarginine in Response to Recombinant Tissue-Type Plasminogen Activator and Erythropoietin in Acute Stroke

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Background and Purpose—In the German Multicenter Erythropoietin (EPO) Stroke Trial, patients not receiving thrombolysis most likely benefited from EPO on clinical recovery, whereas a combination of rtPA and EPO was associated with increased mortality. We investigated whether the combination of rtPA and EPO increased release of the endogenous NO synthase inhibitor asymmetric dimethylarginine (ADMA), and thereby potentially deteriorated ischemic stroke outcome, as suggested from experimental data.

Methods—ADMA was determined in serum samples from 90 patients of the German Multicenter EPO Stroke Trial taken at days 1 (within 6 hours after symptom onset), 2, 3, 4, and 7 after stroke using high-performance liquid chromatography–tandem mass spectrometry. ADMA was analyzed for the different treatment groups (EPO, n=25; placebo, n=30; rtPA+placebo, n=18; EPO+rtPA, n=17). Clinical outcome was expressed as difference between National Institutes of Health Stroke Scale at baseline and 90 days.

Results—ADMA levels significantly increased during the observation time in EPO, EPO+rtPA, and placebo groups (P<0.05). A treatment effect on ADMA levels was revealed by repeated measures ANOVA only in the rtPA+placebo group (P=0.027). Here, ADMA levels were decreased compared with the placebo group (P<0.05). Both the EPO and the rtPA+placebo groups in the Hannover subgroup of the EPO trial had better outcome than the placebo group (P<0.05).

Conclusions—Our data underscore the potential benefit of EPO in ischemic stroke. The hypothesis from experimental data, that EPO treatment increases ADMA in stroke patients, was disproved. Further studies are needed to clarify whether decreased ADMA might contribute to therapeutic rtPA effects. (Stroke. 2013;44:2128-2133.)

Key Words: asymmetric dimethylarginine ■ clinical trial ■ erythropoietin ■ ischemic stroke ■ rtPA

Erythropoietin (EPO) has been demonstrated to have a positive effect on lesion size and outcome in various experimental stroke models.1-3 The Göttingen EPO Stroke Study was the first to explore the effects of EPO in patients with acute ischemic stroke. Set up as a monocentric phase II trial, evaluating safety and potential efficacy, this study suggested that intravenous treatment with EPO improves clinical outcome in acute ischemic stroke.4 The subsequent German Multicenter EPO Stroke Trial (ClinicalTrials.gov; Unique Identifier:NCT00604630) showed a significantly improved outcome in the subgroup of patients treated with EPO compared with those treated with placebo but, nevertheless, the trial turned out as formally negative. This negative outcome was mainly because of the patient group that had received EPO after thrombolysis treatment with recombinant tissue-type plasminogen activator (rtPA).5,6

Subsequently, the effect of the combination of EPO+rtPA has been investigated in experimental stroke models to find potential mechanisms of harm.7,8 In a transient middle cerebral artery occlusion model it was observed that treatment with the combination of EPO+rtPA reduces the amount of reperfusion in the lesioned hemisphere.8

One candidate substance that could be involved in this process is asymmetric dimethylarginine (ADMA). ADMA is an...

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endogenous NO synthase inhibitor. ADMA increase after acute stroke is associated with adverse outcome.9–11 It is suggested that ADMA might significantly contribute to brain injury after stroke by decreasing cerebral perfusion and triggering oxidative stress and inflammation.12–14 Treatment with EPO increased ADMA levels in both, cell cultures and experimental animals.15,16 We, therefore, hypothesized that EPO administration in acute ischemic stroke patients treated with rtPA might increase ADMA serum levels, thereby potentially deteriorating stroke outcome.

Patients and Methods

The present study is an exploratory subgroup analysis based on the patients of the randomized, double-blind, placebo-controlled German Multicenter EPO Stroke Trial5 who were recruited at Hannover Medical School (n=126). Patients were included into this analysis when all 5 of 5 follow-up blood samples had been drawn (n=90). Reasons for incomplete blood sampling (n=36) were death (n=5), early dropout (n=4), no personnel to take additional samples (n=27). Inclusion and exclusion criteria for the German Multicenter EPO Stroke Trial are listed below.

Inclusion Criteria

Patients aged ≥18 years with ischemic stroke in the middle cerebral artery territory, scoring ≥4 in National Institutes of Health Stroke Scale (NIHSS) scores and time window of ≤6 hours from onset of symptoms to study drug infusion (time to treatment) were included. Acute stroke was confirmed using diffusion-weighted imaging MRI. Fluid-attenuated inversion recovery should essentially be free of fresh infarct signs (or at least show distinctly smaller lesion size compared with diffusion-weighted imaging) and rule out recent infarcts in the same territory.

Exclusion Criteria

Exclusion criteria were contraindications to MRI, fast resolving neurological symptoms, unclear time point of symptom onset, NIHSS ≥1a ≥2, brain trauma/surgery within the last 4 weeks, subarachnoid/intracerebral hemorrhage, intracranial neoplasia, septic embolism, endocarditis, malignant hypertension, florid malignancy, myeloproliferative disorder, antibodies or allergy against EPO, pregnancy, and participation in other treatment trials.

For study treatment patients received intravenous infusion of recombinant human EPO (Epoetin-alpha, provided by J&J, 40000 IU in 50 mL isotonic electrolyte solution >30 minutes) or placebo (solvent control, provided by J&J). Treatment was started within 6 hours after symptom onset (day 1) and repeated 24 and 48 hours later. Patients were treated according to the guidelines in stroke therapy effective at that time; thereby 35 patients who were admitted within 3 hours after stroke received rtPA treatment.

For the present study, patients were grouped into different treatment groups comprising study treatment with EPO and rtPA treatment according to the clinical guidelines (EPO, n=25; rtPA+placebo, n=18; EPO+rtPA, n=17; placebo, n=30). Clinical outcome was expressed as difference between NIHSS at baseline and at 90 days.

Clinical Assessment

Clinical data recorded from all patients included demographic characteristics (age, sex), baseline stroke severity (NIHSS on admission), stroke subtype classified according to Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria, systolic and diastolic blood pressure, the stroke risk factors hypertension, diabetes mellitus, hyperlipidemia and smoking status, routine laboratory (eg, hemoglobin at admission), status of infection at admission, and the clinical outcome scales NIHSS, modified Rankin Scale, and Barthel Index at 90 days.

The study was approved by the ethics committee of the Georg-August-University of Göttingen and the ethics committee of Hannover Medical School. Patients or a guardians gave written informed consent.

Blood Collection and Measurement of ADMA

Venous blood samples were taken at days 1 (within 6 hours after symptom onset), 2, 3, 4, and 7 after stroke. Serum was stored at −80°C until assayed. Serum ADMA was assessed using high-performance liquid chromatography–tandem mass spectrometry.17 The measurement was performed blindly without knowledge of any of the clinical information. For preparation of samples, proteins were precipitated and the composition of the samples and the high-performance liquid chromatography system mobile phase consisting of water–acetonitrile–trifluoroacetic acid–propionic acid was equalized. Samples were injected into the high-performance liquid chromatography–tandem mass spectrometry system and separated by hydrophilic interaction liquid chromatography. After electrospray ionization ADMA was detected by tandem mass spectrometric single-ion monitoring. The lower limits of quantification for ADMA were 0.15 μmol/L. The intraassay precision was 3.8% and the intra-assay precision was 2.1%.

Statistical Analysis

Data were analyzed using the IBM SPSS statistics version 19.0. For the analysis, patients were grouped into different treatment groups. Data are presented as numbers and portion for categorical variables and median with interquartile range for continuous variables. The data were tested for statistically significant differences between treatment groups by Kruskal–Wallis test and Mann–Whitney U test for continuous data and Pearson χ² for categorical data. In addition, outcome as expressed by ∆NIHSS was compared between patient groups by binary logistic regression (covariates age, infection at day 1) and presented as means/SD.

Within-group comparisons of ADMA levels at different time points were analyzed by Wilcoxon test. Treatment effects on ADMA were tested by 2×2 repeated measures analysis adjusting for age and infection at day 1. Spearman correlation was used to study the correlation between ADMA levels and ∆NIHSS. A P value of <0.05 was considered to indicate statistical significance.

Results

Clinical characteristics of the patient groups stratified by treatment are shown in the Table. The baseline characteristics, such as cardiovascular risk factors did not significantly differ between the groups, but patients who underwent thrombolysis treatment tended to be younger and status of infection at day 1 tended to differ.

Of note, patients excluded from the study, because not all 5 of 5 follow-up blood samples had been drawn, did not differ from the included study cohort with regard to baseline characteristics (Table I in the online-only Data Supplement).

Time Course of ADMA in Different Treatment Groups

ADMA levels significantly increased during the observation time in patients who received EPO, EPO+rtPA, or placebo (P<0.05; Figure 1; Table II in the online-only Data Supplement). Of note, ADMA levels remained unchanged in the rtPA+placebo group.

For the time course of ADMA levels grouped by different treatment, we observed no difference between patients who received EPO and those who received placebo. But those patients who were treated with rtPA showed lower levels of ADMA at each time point between days 2 and 7, independent of whether the comedication was EPO or placebo (P<0.05; Figure 1).

Therefore, treatment effects on the change in ADMA levels were tested by 2×2 repeated measures analysis adjusting
for age and infection at day 1. A significant treatment effect was revealed only in the rtPA+placebo group (P=0.027).

Change in ADMA levels was significantly lower for patients treated with rtPA+placebo compared with patients who received placebo (Mann–Whitney U test: days 1–2, P=0.007; days 1–3, P<0.001; days 1–4, P=0.001; and days 1–7, P=0.014).

## Clinical Outcome in Treatment Groups

We further evaluated whether these differences in ADMA levels were accompanied by differences in clinical outcome. Analysis of the clinical course of the 4 subgroups showed significant differences for NIHSS at day 90 (P=0.002), ΔNIHSS (difference NIHSS at baseline and at 90 days; rtPA, recombinant tissue-type plasminogen activator; and SBP, systolic blood pressure at day 1 (before treatment start).

### Correlation of ADMA Levels and Clinical Outcome

Change in ADMA levels from admission to the days to follow-up was significantly correlated with ΔNIHSS (ΔADMA

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### Figure 1. Temporal profile of asymmetric dimethylarginine (ADMA) by treatment groups. Temporal evolution of ADMA levels in acute ischemic stroke patients who received erythropoietin (EPO), recombinant tissue-type plasminogen activator (rtPA)+placebo, EPO+rtPA, or placebo. ADMA was measured between day 1 and day 7 after onset of symptoms. Data are presented as median and interquartile range. Within-group comparisons for ADMA levels between baseline and follow-up time points: significant differences were detected for treatment with EPO (P<0.05), EPO+rtPA (%P<0.05), and placebo (#P<0.05). ADMA levels remained unchanged in the rtPA+placebo group.
days 1–2: \( r=0.218, P=0.039 \); ΔADMA days 1–3: \( r=0.348, P=0.001 \); ΔADMA days 1–4: \( r=0.300, P=0.004 \); and ΔADMA days 1–7: \( r=0.309, P=0.003 \); Figure 3).

For analysis of the different treatment groups, a significant correlation between ΔNIHSS and the difference in ADMA levels was detected only in the rtPA-treated patients (Δdays 1–2: \( r=0.504, P=0.039 \)).

**Discussion**

In the present study, we investigated for the first time the course of ADMA levels >5 days after ischemic stroke on different treatment regimens. Patients were treated with EPO, rtPA+placebo, EPO+rtPA, or placebo. The pertinent findings of our study are (1) ADMA levels increased during the observation time in the EPO, the EPO+rtPA, and the placebo group. (2) A treatment effect on ADMA levels was revealed only in the rtPA+placebo group when adjusted for age and infection at day 1. Here, ADMA levels were significantly decreased compared with the placebo group. (3) The Hannover subgroup of this trial revealed better outcome of both EPO- and rtPA+placebo-treated groups compared with placebo. These findings require further consideration.

**No Augmentation of ADMA Increase by Treatment With EPO in Acute Stroke**

In acute ischemic stroke oxidative stress and inflammation may cause increased production of ADMA by expression of protein arginine methyltransferases and decreased metabolism of ADMA by low enzymatic activity of dimethylaminohydrolases. Increased ADMA levels are suggested to cause impairment of brain perfusion because of decreased NO concentration. Our current data revealed that in ischemic stroke patients, who were treated with EPO, EPO+rtPA, or placebo, ADMA levels significantly increase in the first days after stroke. This is in line with previous data from a clinical stroke study that did not include subgroup analysis for acute stroke treatment. Considering data from animal models and endothelial cell cultures, we hypothesized that elevation of ADMA levels might potentiate NO increase after acute stroke, contributes to rtPA treatment effects in acute stroke. In healthy controls, infusion of ADMA increased arterial stiffness and decreased cerebral blood flow, suggesting that ADMA decrease in rtPA-treated patients is able to enhance cerebral blood flow. Because postinfusion levels of ADMA were 15.41 (±4.39) µmol/L, one might wonder whether the relatively small changes in ADMA levels in the current study may be of relevance for cerebral blood flow. Experimental data showed further that local administration of 10 µmol ADMA markedly impaired responses of cerebral arterioles to Ach. In mice with overexpression of dimethylaminohydrolase-1, which catalyzes ADMA, this effect was almost abolished indicating a prominent effect of ADMA in cerebral arterioles.

**rtPA Treatment Decreases ADMA**

Our data showed a significant and independent effect for rtPA treatment on ADMA levels because stroke patients who were treated with rtPA+placebo showed significantly lower ADMA levels than patients who received placebo. It remains unclear whether this decrease of ADMA levels, which might potentiate NO increase after acute stroke, contributes to rtPA treatment effects in acute stroke. In healthy controls, infusion of ADMA increased arterial stiffness and decreased cerebral blood flow, suggesting that ADMA decrease in rtPA-treated patients is able to enhance cerebral blood flow. Because postinfusion levels of ADMA were 15.41 (±4.39) µmol/L, one might wonder whether the relatively small changes in ADMA levels in the current study may be of relevance for cerebral blood flow. Experimental data showed further that local administration of 10 µmol ADMA markedly impaired responses of cerebral arterioles to Ach. In mice with overexpression of dimethylaminohydrolase-1, which catalyzes ADMA, this effect was almost abolished indicating a prominent effect of ADMA in cerebral arterioles.

However, rtPA effects by increase of NO have been controversially discussed because massive increase of NO after ischemic stroke induces neurotoxicity. Thus, at this point the suggested mechanisms remain preliminary conclusions and need to be replicated in further studies.

For a better understanding of possible interactions between ADMA and thrombolyis therapy with rtPA, it must be considered that rtPA for thrombolytic therapy in ischemic stroke is administered in the formulation of alteplase, a combination of rtPA and a stabilization solution of L-arginine. L-Arginine
is a substrate for NO synthesis. A time-dependent effect of \(\text{l-arginine}\) on the brain injury after stroke has been suggested because early administration of \(\text{l-arginine}\) might be beneficial by increase of cerebral blood flow,\(^{21}\) whereas late administration might induce neurotoxicity by massive increase of NO.\(^ {22}\) Eventually, in the current study in those patients treated with thrombolytic therapy, both, intravenous administration of \(\text{l-arginine}\) and decreased levels of ADMA, should increase production of NO. Of note, outcome was improved in these patients. However, from a clinical study, in healthy individuals aged >70 years there is no evidence for a direct link between administration of \(\text{l-arginine}\) and ADMA because ADMA levels remained unchanged after oral administration of \(\text{l-arginine}.^{24}\)

**Acute Stroke Treatment, ADMA, and Outcome**

Previous data from our group and others indicate that ADMA levels are associated with clinical outcome and survival after ischemic stroke, although it remained unclear whether ADMA increase contributes to adverse outcome.\(^ {9,25,26}\) Also in the current study, ADMA levels and additionally the change in ADMA levels are related to clinical outcome. However, this association of ADMA levels with outcome was not detected when patients were grouped by treatment except for a correlation between an increase of ADMA from day 1 to 2 and adverse outcome in rtPA+placebo-treated patients. This might further point to a role for decreased ADMA levels in rtPA treatment effects.

At any rate our data disprove the hypothesis that an increase in ADMA levels with thrombolysis plus EPO therapy might have induced worse outcome in this therapy subgroup in the German Multicenter EPO Stroke trial. A simple causative relationship between ADMA levels and outcome after ischemic stroke is questionable because both the rtPA+placebo-treated subgroup and the EPO-treated subgroup in this study showed a significantly improved outcome compared with controls, although ADMA levels were significantly lower for the first group and comparable with the placebo group in the latter.

Of note, these data are in line to those of another recently published subgroup analysis of the German Multicenter EPO Stroke Trial in 163 patients indicating that patients nonqualifying for rtPA treatment had a significantly better outcome under EPO as compared with placebo treatment. Decreased levels of biomarkers reflecting the degree of brain damage supported this finding.\(^ {27}\)

**Limitations**

Despite being the first clinical study investigating the effect of acute stroke treatment on ADMA levels, our study has some limitations. The number of patients is rather small, considering that the included 90 patients are stratified into 4 treatment groups, but we were able to analyze serial blood samples from all these patients. Of note, the patients included in this analysis were representative for the whole sample recruited for the study at Hannover Medical School (n=126). Another limitation is the assessment of ADMA levels in venous blood because concentrations of ADMA at the site of infarction may only partially be reflected by the ADMA response in the peripheral blood. However, cerebrospinal fluid samples would be difficult to obtain in a longitudinal fashion. In addition, although our data suggest a clear association between ADMA and NIHSS, used as surrogate for stroke outcome, it has to be pointed out that ischemic stroke may cause symptoms that are not accurately reflected by the NIHSS score. Finally, the observational character of the study by analysis of blood samples does not allow the elucidation of pathophysiologic concepts (eg, clarify how rtPA decreases levels of ADMA). This should be addressed in further studies.

**Conclusions**

We disproved the hypothesis from experimental models that ADMA levels are increased in stroke patients treated with EPO if compared with levels in placebo-treated patients. Also, combined treatment of EPO+rtPA did not increase ADMA levels compared with placebo-treated patients. Furthermore, patients showed beneficial outcome when treated with EPO in comparison with the placebo group, underscoring the potential benefit of EPO in acute ischemic stroke. A treatment effect on ADMA levels was revealed in the rtPA+placebo-treated patient group as ADMA levels were lower compared with the placebo-treated group. Further studies are needed to clarify whether decreased ADMA levels might contribute to therapeutic rtPA effects by reduction of oxidative stress and NO-induced prevention from hypoperfusion.

**Sources of Funding**

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

**Disclosures**

Dr Kielstein runs and hosts the website www.adma.com. Dr Ehrenreich holds/has submitted patents on the use of erythropoietin for treatment of cerebral ischemia, schizophrenia and multiple sclerosis.

**References**


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SUPPLEMENTAL MATERIAL

Asymmetric dimethylarginine in response to recombinant tissue plasminogen activator and erythropoietin in acute stroke

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Cover title: ADMA in response to rtPA and EPO after stroke

Key words: erythropoietin, rtPA, asymmetric dimethylarginine, ischemic stroke, clinical trial
Table SI: Baseline characteristics of patients included/ excluded in the study

<table>
<thead>
<tr>
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<th>Patients included in the study (n = 90)</th>
<th>Patients excluded from the study (n = 36)</th>
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<tr>
<td>Female</td>
<td>46 (51.1)</td>
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<td>19 (52.8)</td>
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<td>73 (59; 79)</td>
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<tr>
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<td>155 (136; 176)</td>
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<td>DBP 1d [mmHg]</td>
<td>88 (78; 95)</td>
<td>86 (76; 98)</td>
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<td>Hb 1d [g/dl]</td>
<td>13.6 (12.5; 14.9)</td>
<td>13.6 (12.4; 15.7)</td>
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<td>NIHSS 1d</td>
<td>12 (9; 18)</td>
<td>13 (10; 19)</td>
<td>0.577</td>
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<td>Infection 1d</td>
<td>11 (12.2)</td>
<td>3 (8.3)</td>
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Patients were excluded from the study, because not all 5 out of 5 follow-up blood samples had been drawn. NIHSS = National Institutes of Health Stroke Scale; SBP = Systolic blood pressure at day 1 (before treatment start); DBP = diastolic blood pressure at day 1 (before treatment start), Hb = hemoglobin. Data are presented as numbers (percentages) or median (interquartal range). P<0.05 was considered statistically significant.
<table>
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<th>Placebo (n = 30)</th>
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<th>rtPA + placebo (n = 17)</th>
<th>EPO+rtPA (n = 18)</th>
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<td>ADMA 1d [μmol/l]</td>
<td>0.647 (0.581; 0.850)</td>
<td>0.743 (0.574; 0.918)</td>
<td>0.706 (0.504; 0.756)</td>
<td>0.589 (0.463; 0.760)</td>
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<td>ADMA 2d [μmol/l]</td>
<td>0.852 (0.666; 1.076)</td>
<td>0.899 (0.621; 1.119)</td>
<td>0.711 (0.567; 0.825)</td>
<td>0.641 (0.588; 0.855)</td>
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<td>ADMA 3d [μmol/l]</td>
<td>0.733 (0.681; 1.068)</td>
<td>0.846 (0.662; 0.977)</td>
<td>0.615 (0.535; 0.769)</td>
<td>0.596 (0.524; 0.966)</td>
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<tr>
<td>ADMA 4d [μmol/l]</td>
<td>0.718 (0.597; 1.077)</td>
<td>0.754 (0.664; 0.982)</td>
<td>0.617 (0.493; 0.756)</td>
<td>0.586 (0.482; 0.859)</td>
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<tr>
<td>ADMA 7d [μmol/l]</td>
<td>0.718 (0.591; 0.961)</td>
<td>0.763 (0.662; 0.986)</td>
<td>0.617 (0.506; 0.738)</td>
<td>0.564 (0.484; 0.890)</td>
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<tr>
<td>Delta ADMA day 2-1</td>
<td>0.169 (0.083; 0.258)</td>
<td>0.081 (0.021; 0.229)</td>
<td>0.064 (-0.008; 0.157)</td>
<td>0.085 (0.049; 0.152)</td>
<td>0.026</td>
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<tr>
<td>Delta ADMA day 3-1</td>
<td>0.167 (0.041; 0.300)</td>
<td>0.045 (-0.018; 0.208)</td>
<td>-0.039 (-0.169; 0.695)</td>
<td>0.092 (-0.011; 0.168)</td>
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<td>Delta ADMA day 4-1</td>
<td>0.135 (0.000; 0.255)</td>
<td>0.040 (-0.045; 0.150)</td>
<td>-0.020 (-0.100; 0.050)</td>
<td>0.030 (-0.040; 0.123)</td>
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</tr>
<tr>
<td>Delta ADMA day 7-1</td>
<td>0.108 (0.007; 0.220)</td>
<td>0.011 (-0.052; 0.115)</td>
<td>-0.024 (-0.108; 0.062)</td>
<td>0.024 (-0.072; 0.120)</td>
<td>0.070</td>
</tr>
</tbody>
</table>

ADMA levels were measured at the indicated time points after symptom onset. Data of ADMA and delta ADMA are presented as median (interquartile range). P<0.05 was considered statistically significant.