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Monitoring of L-Arginine and Endogenous Dimethylarginines in Survivor Septic Patients – A Pilot Study

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Abstract. *Background/Aim: Nitric oxide (NO) pathway plays a major role in the development and advancement of inflammation. We aimed to design a study and investigate its feasibility to show the changes of L-arginine, asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA), which are important regulators of the NO pathway. Patients and Methods: Concentrations of L-arginine, ADMA and SDMA were measured by liquid chromatography-tandem mass spectrometry. Seventeen septic survival patients were enrolled and blood samples were obtained on the first, third and fifth day after the diagnosis of sepsis. Sixteen non-septic matched controls were recruited. Results: ADMA levels on admission correlated well with sequential organ failure assessment (SOFA) score. During the follow-up, L-arginine/ADMA ratio increased significantly from day 1 to day 3 (p=0.005), then decreased from day 3 to day 5 (p=0.023). Conclusion: This study design seems feasible to investigate changes of L-Arginine, ADMA and SDMA in sepsis survival patients.*

Sepsis syndrome remains one of the most challenging healthcare issues worldwide. The prominently high mortality rate (approximately 30%) and costs of care (22,000 USD/case) makes it a remarkable disease (1). Septic shock belongs to the leading causes of death in intensive-care units (ICU), even nowadays (2). Early diagnosis and goal-directed therapy are essential for favorable outcome (3). Beside the clinical signs and symptoms, laboratory parameters are essential in proper decision-making. Monitoring the septic process is an essential part of successful therapy; therefore, biomarkers with predictive capacity would be of utmost importance, which are unfortunately not available in current management of sepsis.

Nitric oxide (NO) is a crucial mediator in the inflammatory activation process (4). During sepsis, NO overproduction plays a major role in the development of hemodynamic instability and organ failure. Due to uncoupling of nitric oxide synthase (NOS), excessive production of free radicals, particularly peroxynitrite, leads to oxidative cell injury. Previous studies have shown that NOS inhibitors like endogenous dimethylarginines take considerable role in sepsis by modulating NO related biochemical pathways (5).

Dimethylarginines are produced as a result of protein degradation; methylation occurs by the protein arginine methyltransferase enzyme type 1 and 2 (PRMT-1, PRMT-2). Asymmetric dimethylarginine (ADMA) is produced by PRMT 1, while symmetric dimethylarginine (SDMA) by PMRT-2 (6, 7). SDMA levels are mainly modulated by urinary excretion; however, certain metabolic routes have been found to correspond with its regulation (8). Only 20% of ADMA is secreted into urine and it – but not SDMA – is mainly metabolized by dimethylarginine dimethylaminohydrolase enzyme (DDAH). DDAH-1 is expressed in neuronal tissues, while DDAH-2 is mainly found in endothelial cells. Expression and function of DDAH is impaired by increased glucose, hypercholesterinemia, hyperhomocysteinemia and

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inflammatory milieu (6, 9). Loss of efficiency of DDAH is directly proportional to the severity of oxidative stress (10).

L-arginine is converted by endothelial nitric oxide synthase (eNOS) to L-citrulline and NO. The crucial role of NO in vascular homeostasis and in organ perfusion is well known. Several factors are capable of impairing the beneficial effects of NO. One of them is ADMA, which is an endogenous competitive inhibitor of NOS. Case-control studies have found elevated ADMA concentrations among patients with classic cardiovascular risk factors (*e.g.* smoking, hypertension, obesity, diabetes mellitus, *etc.*) (5, 6). Elevated ADMA levels reduce vascular reactivity and increase peripheral resistance irrespective of disease-related or externally administered drugs influencing vascular response (11, 12). Due to its biological functions, ADMA is considered to be a novel cardiovascular (CV) risk factor. In the case of CV diseases, the clinical significance of ADMA is very well outlined. ADMA has been proven to play a prominent role in atherogenesis; furthermore, it is a marker of endothelial dysfunction. Based on large cohort studies, ADMA is capable of predicting end-points of CV diseases independently (13-18). Until recently, SDMA was considered a functionally inactive isomer of ADMA; however, nowadays, it is found to compete with L-arginine in the reaction with NOS. Therefore, it can decrease NO indirectly. Moreover, overproduction of both ADMA and SDMA were found to increase the formation of reactive oxygen species (11, 19).

This pilot study aimed to test the feasibility of the study design in monitoring the changes of L-arginine and dimethylarginines in early sepsis. Furthermore, the Authors aimed to identify a possible alteration pattern of the mentioned biomarkers in sepsis survival patients, and describe their informational value on disease severity and sepsis-related organ dysfunctions.

Patients and Methods

Study design. The present study was performed at our multidisciplinary adult ICU from January 2015 to April 2015. Severe septic patients were enrolled and followed- up for 5 days. Severe sepsis was defined as recommended by the current consensus and guidelines (20, 21). Inclusion criteria were survival of ICU stay, sepsis-induced organ dysfunction, hypoperfusion abnormalities or hypotension and procalcitonin levels of >2 ng/ml. The first sample was obtained within 24 h after clinical diagnosis and further samples were taken on the 3rd and 5th days, respectively. Sequential organ failure assessment (SOFA) scoring system was used to describe organ dysfunctions (22).

The study protocol was approved by the Regional Ethics Committee of University of Pécs (permission No.: 4327.316-2900/KK15/2011.) in accordance with the 2008 Helsinki declaration. Informed written consent was obtained from every patient.

Exclusion criteria were history of chronic kidney disease, acute myocardial infarction (AMI), stroke, likelihood risk of death due to primary disease and withdrawal of consent.

Age- and gender-matched patients with similar medical history were recruited as control group. Only one sample was obtained from the control patients.

Sampling and obtaining routine laboratory and clinical data. Daily routine parameters (white blood cells (WBC), high-sensitivity C-reactive protein (hs-CRP), procalcitonin (PCT), lactate dehydrogenase (LDH), creatinine, urea, *etc.*) were measured in our university clinical laboratory on automated analyzers by manufacturer's protocol (Cobas 8000; Roche Diagnostics GmbH, Mannheim, Germany). Clinical data, like organ dysfunction parameters (blood pressure, urine output, drugs, *etc.*), were registered daily. For the assessment of disease severity and mortality prediction, the simplified acute physiology score II (SAPS II), the acute physiology and chronic health evaluation II (APACHE II) and the SOFA scores were calculated. Patients surviving ICU were considered to be survivors.

Blood samples were taken using 3.8% sodium-citrate BD Vacutainer® Blood Collection tubes (Becton Dickinson, Franklin Lakes, NJ, USA) both from septic and control patients besides the daily blood collection for routine laboratory tests. After centrifugation (1,500 × *g*, 10 min) plasma was collected and stored at –70˚C until analysis.

Measurement of L-arginine and dimethylarginines. After sample preparation, L-arginine, ADMA and SDMA levels were determined by liquid chromatography-tandem mass spectrometry method described by Martens-Lobenhoffer *et al.* (23). Minor modifications were implemented to transfer the method to our Dionex Ultimate 3000 HPLC/Thermo Q Exactive setup (Thermo Fisher Scientific, Waltham, MA, USA). Target substances were separated using a Kinetex HILIC column (product no.: 00D-4461-AN; Phenomenex, Torrance, CA, USA). Data obtained in MS^2 mode were extracted by pseudo-MRM method using the "Xcalibur 2.2 Qual Browser" evaluation software (Thermo Fisher Scientific).

L-arginine monohydrochloride (product no.: A5131; Sigma Aldrich, Saint Louis, MO, USA), N^G,$N^{G'}$-Dimethyl-L-arginine di(p-hydroxyazobenzene-p'-sulfonate) (product no.: D0390; Sigma Aldrich) and N^G,N^G Dimethylarginine dihydrochloride (product no.: D4268; Sigma Aldrich) standards were used for calibration.

The quantitation range was 0.073-37.5 μM for ADMA and SDMA and 1.172-150 μM for L-arginine. The calibration models used in these ranges were non-linear regarding that the ion trap instruments have narrow linear quantitative range. The regression coefficient was R^2>0.995 and the precision was lower than 10% relative standard deviation (RSD) for all compounds in case of the calibrations. The accuracy was ± =15% for the quality control (QC) samples (spiked plasma).

Statistical analysis. Statistical analysis was performed by IBM SPSS Statistics for Windows Version 22 (IBM Corp, Armonk, NY, USA). Sample normality was checked by Shapiro-Wilk test. In the case of variables showing normal distribution (ADMA), independent sample t-test was used to compare two groups and one-way ANOVA was performed to compare more than two groups. Data acquired during the follow-up study were analyzed with repeated measures ANOVA tests.

Non-parametric tests were used to compare non-normally distributed variables (L-arginine, SDMA). For comparison of our groups, Mann-Whitney *U*-test (two groups) or Kruskal-Wallis test

Table I. *Demographic data of the involved patients.*

	Control group	Septic patients
Number of patients	16	17
Mean age, years (±SD)	61 (13)	66 (15)
Male, n (%)	9 (56)	8 (47)
Hypertension, n (%)	13 (81)	14 (82)
Pulmonary disease, n (%)	6 (38)	8 (47)
Diabetes Type 2, n (%)	4 (24)	4 (24)

Table II. *Clinical characteristics of septic patients. Median data and IQR are presented.*

	Septic patients
Type of admission	
surgical, n (%)	11 (65)
non-surgical, n (%)	6 (35)
Length of ICU stay, days (IQR)	7 (3-8)
SOFA score, (IQR)	7 (5-9)
SAPS II score, (IQR)	39 (34-51)
APACHE II score, (IQR)	16 (12-23)

ICU, Intensive care unit; SOFA, sepsis-related organ failure assessment; SAPS, simplified acute physiology score; APACHE, acute physiology and chronic health evaluation; IQR, interquartile range.

(more than two groups) were carried out, while differences during the follow-up were investigated by Friedman's two-way ANOVA. To reveal correlations, Spearman's Rank Order Test was used. All *p*-values lower than 0.05 were considered as statistically significant. Continuous variables are presented as medians with quartiles (25-75%).

Results

Clinical characteristics and disease outcome of participants. In the present study, 17 severe septic patients and 16 age-, gender- and medical history-matched control individuals were enrolled. Demographic data are represented in Table I. Among 17 septic patients, 12 suffered from septic shock, the remaining 5 patients developed sepsis-induced organ dysfunctions. In four cases of sepsis, no microbes were identified, 2/2 patients suffered from isolated Gram-positive or Gram-negative bacteria, only one patient had fungal infection and mixed microbial infections were identified in eight patients. Clinical characteristics of involved patients are shown in Table II.

The baseline of controls and follow-ups of septic patients ADMA, SDMA, L-arginine, ADMA/SDMA ratio and L-arginine/ ADMA ratio are shown in Figures 1 and 2. Significant biochemical parameters are shown in Table III.

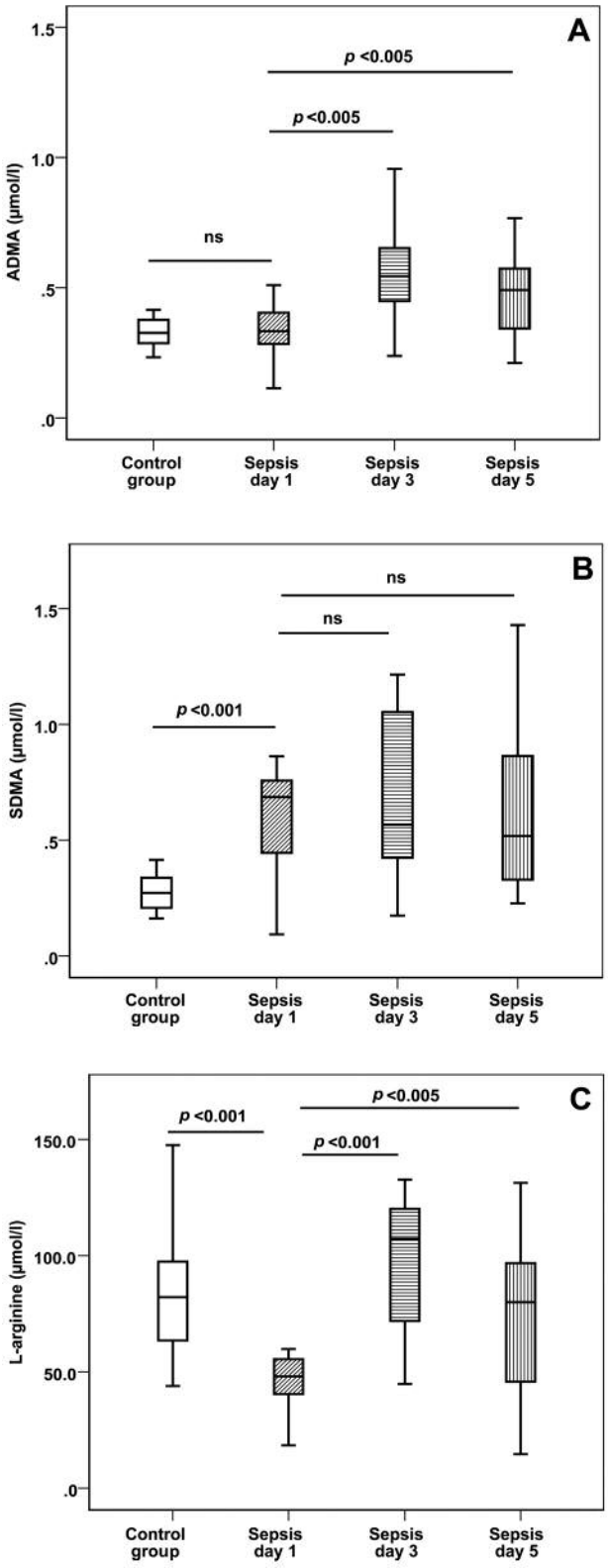

Figure 1. *Monitoring of endogenous dimethylarginines (A, B) and L-arginine (C) in sepsis. ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; ns, non-significant.*

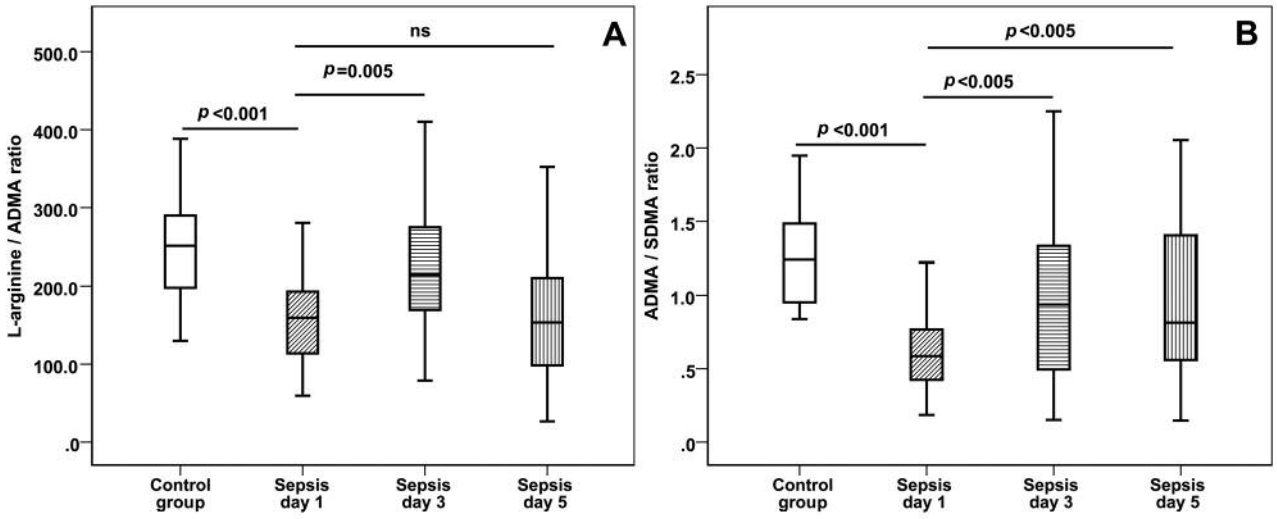

Figure 2. *L-arginine/ADMA ratio (A) and ADMA/SDMA ratio (B) in sepsis. ADMA, asymmetric dimethylarginine; SDMA, symmetric dimethylarginine; ns, non-significant.*

Table III. *Baseline biochemical parameters of controls and follow-ups of septic patients.*

	Control group	Septic patients		
		Day 1	Day 3	Day 5
WBC count (G/l)	7.64 (5.99-8.23)	12.90 (9.22-17.83)	11.70 (8.77-14.37)	10.67 (8.77-15.68)
Neutrophil count (G/l)	4.78 (3.37-5.83)	11.32 (7.39-16.51)	10.75 (7.59-12.61)	9.27 (6.32-13.02)
Lactate (mmol/l)	–	2 (1.2-2.6)	1 (0.725-1.4)	1.25 (0.875-1.375)
Total protein (g/l)	72.3 (68.9-75.5)	46.8 (42.5-50.3)	49 (45.1-52)	48.55 (43.3-52.3)
LDH (U/l)	370 (348-406)	548 (379-702)	566 (423-690)	423 (362-535)
hs-CRP (mg/l)	1.91 (0.6975-4.74)	182.00 (124.40-260.61)	152.8 (106.55-210.4)	67.45 (36.79-129.28)
PCT (ng/ml)	–	4.49 (2.39-11.17)	1.74 (0.987-5.78)	1.01 (0.52-2.05)
Urea (mmol/l)	5.20 (4.50-5.975)	7.88 (6.40-12.27)	9.22 (7.03-15.23)	10.8 (6.825-18.22)
Creatinine (μmol/l)	63.00 (56.75-72.25)	104.00 (76.00-131.00)	103 (55-149)	81 (52-151.2)

WBC, White blood cells; LDH, lactate dehydrogenase; hs-CRP, high-sensitivity C-reactive protein; PCT, procalcitonin. Data are presented as median and interquartile range (IQR). Lactate and PCT were not measured in controls.

Both hs-CRP and PCT showed decreasing tendency during the follow-up period. Hs-CRP and PCT levels decreased significantly from day 1 to day 3 ($p<0.005$), further decrease was found from day 3 to day 5 ($p<0.005$).

ADMA, survival and disease severity. There were no significant differences in ADMA concentrations between controls and septic patients on the first follow-up day. However, ADMA was significantly higher in septic patients on day 3 ($p=0.001$) and day 5 ($p=0.003$) compared to controls. During the follow-up, ADMA increased significantly from day 1 to day 3 ($p=0.003$); afterwards, on day 5, ADMA decreased, but remained significantly higher than on day 1 ($p=0.027$). Patients suffering from more than

three organ failures had significantly higher ADMA concentrations compared to patients with less than three organ failures (0.573 *vs*. 0.425 μmol/l, p=0.018). Regarding the first follow-up day, higher ADMA concentrations were found in patients with SOFA >10 compared to SOFA <10 patients (0.542 *vs*. 0.307 μmol/l, p=0.004). ADMA levels correlated well with SOFA scores (0.587, p=0.013).

SDMA and acute kidney injury. During the follow-up period, septic patients had significantly higher SDMA concentrations than controls (p<0.001). No significant changes were found between septic patients during the follow-up period. Patients who developed acute kidney injury during sepsis had significantly higher SDMA concentrations compared to patients without kidney injury (0.81 *vs*. 0.43 μmol/l, p<0.001). A strong correlation was found between SDMA and urea (0.732, p<0.001) and SDMA and creatinine levels, respectively (0.793, p<0.001).

L-arginine. On the first follow-up day, lower L-arginine concentrations were measured in septic patients compared to controls (p<0.001). However, a significant elevation was found from day 1 to day 3 in sepsis, (p<0.001); afterwards, L-arginine levels decreased significantly from day 3 to day 5 (p=0.004).

L-arginine/ADMA ratio. Control patients had significantly higher L-arginine/ADMA ratio than septic patients on day 1 (p<0.001); afterwards, the ratio showed an increasing non-significant tendency. Finally, on day 5, the ratio decreased and remained significantly lower compared to controls (p=0.007). We found significantly increased L-arginine/ADMA ratio from day 1 to day 3 (p=0.005). Moreover, on day 5, L-arginine/ADMA ratio decreased significantly compared to day 3 (p=0.023). Patients with sepsis-induced hypoperfusion showed significantly elevated L-arginine/ADMA ratios (p=0.015).

ADMA/SDMA ratio. Control patients had significantly higher ADMA/SDMA ratio than septic patients on day 1 (p<0.001). Regarding the septic patients, significantly increased ADMA/SDMA ratio was found from day 1 to day 3 (p=0.015); afterwards, the ratio levels decreased significantly from day 3 to day 5 (p=0.036).

Discussion

Several studies have investigated plasma dimethylarginine levels in sepsis. However, in some studies, more than 24 h elapsed between sepsis diagnosis and sample collection. Consequently, initial stage of sepsis was missed (24), while others were only limited to two samples (19, 25-27). Only two relevant studies have investigated L-arginine, ADMA and SDMA levels together. One of these used an inaccurate sampling protocol, taking blood samples at the onset of sepsis and between the 2nd and 4th day (25). The other study missed to report the changes of L-arginine and L-arginine/ADMA ratio, thereby lacking the information of NO bioavailability (28). The precise regulation of NO system is essential to survive sepsis. Insufficient production of NO impairs the activity of antimicrobial system. However, uncontrolled NO production can lead to uncontrollable hypotension and massive oxidative stress, that can result in organ failure and death (5). These findings delineate the effects of L-arginine and dimethylarginines on NO system during sepsis. ADMA levels are considered to be associated with sepsis survival due to their significant role in vascular reactivity, microcirculation and organ perfusion (19, 25, 26). This association was confirmed even in this few-patient population by revealing significant correlation between ADMA levels and SOFA score measured and calculated on day 1. Regarding organ dysfunctions, SDMA seems to be an early marker of acute kidney injury, which can be explained by its urinary excretion (6, 28). The initially decreased L-arginine levels shown in Figure 1 are in line with literature and can be explained by massive catabolic state and reduced, *de novo* L-arginine production (29). The causes of increased L-arginine levels during the course of sepsis are controversial. As shown in Figure 1, blood levels of L-arginine and ADMA were elevated significantly from day 1 to day 3, which agrees with the so called "L-arginine paradox". Namely, ADMA levels are high enough to impair the function of eNOS enzyme making it incapable of converting L-arginine to NO, despite the sufficient amount of L-arginine (29-32). Subsequently, both L-arginine and ADMA show non-significant decreasing tendencies but remain, however, still significantly higher than on the onset of sepsis. This can be explained by the efficient treatment indicated by the decreasing tendencies of hs-CRP and PCT and high survival rate. L-arginine/ADMA ratio is considered as an indicator of NO bioavailability. The crucial importance of NO levels in sepsis has been demonstrated in several studies (25, 28-30, 33). Regarding the onset of sepsis, we found decreased L-arginine/ADMA ratio, which can indicate reduced endothelial NO production due to reduced availability of L-arginine to NOS (31, 32). The elevation of L-arginine/ADMA ratio experienced from day 1 to day 3 can be once again attributed to the "L-arginine paradox". Interestingly, L-arginine/ADMA ratio calculated on day 1 to day 5 showed no significant differences. The results provided by Brenner *et al*. showed similar trends in changes of L-arginine/ADMA ratio but with a 2-day right shift in time (33). Due to the high survival rate and decreasing tendencies of PCT and hs-CRP, L-arginine/ADMA ratio shown in Figure 2 could denote a survival pattern. In addition, ADMA/SDMA ratio shows an increasing trend from day 1 to day 3 and from day 3 to day 5, which is very similar to the time course of survivor patients demonstrated by Iapichino *et al*. (28).

Conclusion

This study design seems to be capable to investigate the NO pathway in sepsis through the changes of L-arginine and dimethylarginines. Measuring the levels and interpreting the results by these molecules together might provide valuable information regarding the ongoing septic process. Besides prediction of sepsis outcome, L-arginine/ADMA ratio could be a reliable indicator of successful therapy by providing information on NO bioavailability. Furthermore, dimethylarginines could be new and promising target molecules in sepsis treatment.

Conflicts of Interests

The Authors declare that there are no conflicts of interests regarding the publication of this paper.

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