

## The biological evaluation of ADMA/SDMA and eNOS in patients with ACHF

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## 1. ABSTRACT

The aim of this study was to investigate the effects of acute pharmacological treatment on the plasma levels of L-arginine, asymmetrical dimethylarginine (ADMA), and symmetrical dimethylarginine (SDMA). We also investigated the related effects on endothelial nitric oxide synthase (eNOS) expression and activity and cytochrome c oxidase activity in the primary blood mononuclear cells (PBMCs) isolated from patients with acute congestive heart failure (ACHF). Compared to pre-treatment values, ADMA, SDMA, and L-arginine plasma levels were significantly higher after pharmacological treatment (ADMA, 0.82 *versus* 0.43  $\mu$ M; SDMA, 1.52 *versus* 1.12  $\mu$ M; L-arginine, 1.78 *versus* 1.29  $\mu$ M;  $p < 0.01$ ). In addition, the levels of eNOS expression and activity were decreased after pharmacological treatment, while cytochrome c oxidase activity resulted in higher O<sub>2</sub><sup>-</sup> production. In the PBMCs isolated from patients with acute congestive heart failure (ACHF) and impaired renal function, higher SDMA and ADMA levels were more evident after therapy, as were reduced expression and activity of eNOS. Increased O<sub>2</sub><sup>-</sup> produced after treatment may be involved in impaired recovery of cardiac function associated with higher plasma levels of SDMA.

## 2. INTRODUCTION

One link between conventional risk factors for cardiovascular disease (CVD), malnutrition, and chronic inflammation is that oxidative damage plays an important role in each of the elements that contribute to the pathogenesis of endothelial dysfunction (1). Nitric oxide (NO) produced by nitric oxide synthase (NOS) may cause vasodilatation, and is an important factor in the regulation of systemic blood pressure and local blood flow (2).

Recent studies cited in the literature have widely demonstrated that reduction in the bioavailability of NO can have a determining role in the pathogenesis of vascular damage. Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of NO, has emerged as a novel cardiovascular risk factor for CVD associated with endothelial dysfunction, including type 2 diabetes mellitus (3), coronary artery disease (CAD) (4,5), carotid atherosclerosis (6), and end-stage renal disease (7). Systemic accumulation of ADMA has also been implicated in the pathogenesis of heart failure (HF) (8).

ADMA acts directly on NOS, while its analog, symmetric dimethylarginine (SDMA), is implicated as an

important competitive inhibitor for arginine transport across cell membranes. Accumulating data support a physiologic role for NO signaling in the regulation of cardiac inotropy and relaxation. Impairment of the NO pathway can occur by a decrease in the expression of endothelial nitric oxide synthase (eNOS) or an increase in oxidative stress (9). The synthesis of NO from its precursor, L-arginine, may be altered by the action of a complex series of arginine methylation pathways. These pathways function in the presence of inflammation and oxidative stress through degradation of cellular proteins that contain arginine residues (10). In particular, 2 isoforms of methylarginine have been identified as potent endogenous NOS inhibitors: N-mono-methylarginine (MMA) and its methylation product ADMA (11). Acute congestive heart failure (ACHF) is a syndrome characterized by the inability of the heart to fill with or pump blood due to structural or functional cardiac deficiencies (12), and is responsible for more hospitalizations than all forms of cancer combined (13). ACHF may be caused by myocyte death, myocyte dysfunction, ventricular remodeling, or a combination of these factors (14). Patients with HF were found to have increased circulating levels of ADMA when compared with the healthy controls (15, 16). Although elevated ADMA plasma concentrations have been described in patients with HF (11,16), little is known about the plasma concentrations of SDMA in patients with ACHF.

The aim of this study was to investigate the effects of pharmacological treatment on plasma concentrations of ADMA, SDMA, and L-arginine in patients with ACHF, evaluating a possible correlation with the expression and activity of eNOS, or with an increase of oxidative stress.

### 3. MATERIALS AND METHODS

#### 3.1. Study design

Between March and July 2010, all consecutive patients diagnosed with ACHF (defined as acute and progressive resting dyspnea associated with clinical signs of pulmonary or peripheral congestion requiring hospitalization and treatment with an intravenous diuretic) who were admitted to the Intensive Cardiology Unit of San Camillo De Lellis Hospital (Manfredonia, Italy) were invited to participate in this study, which was approved by the ethics committee of the hospital. After being informed about the nature and purpose of the study, all patients gave their voluntary consent. Eligible subjects were 18–75 years old, with left ventricular ejection fractions (LVEF) of 35%. Subjects were excluded if they had acute cardiac decompensation within the previous 7 days, need for coronary revascularization, acute coronary syndrome, significant primary valvular diseases, or significant hepatic or renal dysfunction. Significant hepatic dysfunction was defined as serum aminotransferase levels above twice the upper limit of normal. Significant renal dysfunction was defined as an estimated glomerular filtration rate (eGFR) of  $30 \text{ mL}/(\text{min} \cdot 1.73 \text{ m}^2)$ .

#### 3.2. Patient population

Twenty-four patients with ACHF (LVEF < 35%) were enrolled in the study. Medical and surgical histories, physical conditions, and medications were recorded.

After inclusion, a heparinized blood sample was drawn from an indwelling arterial line for the determination of baseline ADMA, SDMA, and L-arginine levels. Subsequently, a blood sample was drawn after 1 day of therapy. In addition, laboratory parameters indicating renal function (creatinine, urea) and hepatic function (aspartate aminotransferase (AST), alanine aminotransferase (ALT), and complete hemocytometer examination were determined before and after 1 day of therapy. The therapy for patients with HF consisted of diuretics, digoxin, ACE inhibitors or angiotensin receptor blockers, and nitroglycerin.

#### 3.3. Sample collection, storage, and preparation

Blood samples were collected in polypropylene tubes containing 1 mM EDTA. Samples were stored in an icebox prior to centrifugation at  $3000 \times g$  for 10 min at 4°C. Two-hundred-microliter aliquots of plasma were then transferred into Eppendorf tubes, and plasma samples were either used immediately for extraction or stored in the dark at -80°C until analysis.

#### 3.4. Isolation of human peripheral adherent mononuclear cells

Primary blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation through Ficoll/Hypaque (Pharmacia), suspended (at  $8 \times 10^6$  cells/mL) in RPMI 1640 medium with 10% heat inactivated human serum (Sigma, St. Louis, MO), and seeded in flasks. After incubation for 1 h at 37°C, adherent cells were detached, resuspended (at  $2 \times 10^6$  cells/mL) in medium supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO), and seeded onto 6-well tissue culture plates. Cell viability, determined by trypan blue exclusion, was >99%. The medium and serum had very low lipopolysaccharides (LPS) content, as determined by chromogenic assays using *Limulus ameobocyte lysate* (Whittaker Bioproducts, Walkersville, MD).

#### 3.5. Western blot analysis for eNOS

Determination of eNOS protein levels was performed on 2 series of protein extracts by western blotting (17). Equal amounts of protein (50 µg), quantified spectrophotometrically (Hewlett Packard 8452A, CA) by using the Lowry method, from human peripheral adherent mononuclear cells were separated by electrophoresis on a 7.5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE; BIO-RAD, Hercules, California) and transferred to a nitrocellulose membrane (BIO-RAD, Hercules, California) at 4°C in glycine-methanol buffer. The nitrocellulose membrane was then blocked in Tris-buffered saline [TBS]-milk and incubated overnight in primary anti-human eNOS antibody (1:1000; Santa Cruz Biotech, CA). The membrane was then washed in TBS, and incubated with secondary HRP-conjugated antibody (1:10,000; Pierce, Rockford, IL) for 1 h, washed again, and developed. Primary antibody anti-β-Actin (Sigma, St. Louis, MO) was used as an internal standard. The nitrocellulose membrane

**Table 1.** Demographic details of population study

Patients characteristics	
Age (mean ± SD)	64 ± 6
Sex (M/F)	13/11
BMI (Kg/m <sup>2</sup> )	29.17 ± 3.45
NYHA functional class III	13
NYHA functional class IV	11

**Table 2.** Laboratory data

	Pre-treatment	Post-treatment	P-value
Creatinine (mg/dL)	1.42	1.55	ns
Urea (mg/dL)	83	84	ns
AST	29	30	ns
ALT	27	31	ns
HGB L (g/d)	12.3	12.2	ns
Red cells	4.156.000	4.148.000	ns
White cells	8.360	8.422	ns
Haematocrit	34.32	35.12	ns
LVEF (%)	30.15	36.25	p<0.01

**Table 3.** HPLC results

ADMA (μmol/L)	0.55 ± 0.12	0.72 ± 0.18	p<0.01
SDMA (μmol/L)	1.15 ± 0.24	1.29 ± 0.21	p<0.01
L-arginine (μmol/L)	1.33 ± 0.08	1.78±0.091	p<0.01

was scanned using a computerized densitometric system (Bio-Rad Gel Doc 1000, Milan, Italy).

### 3.6. eNOS activity

Conversion of L-arginine to L-citrulline was measured with a standard assay used to quantify eNOS activity (18). Briefly, 10 μL of radioactive arginine, L-(2,3,4,5-<sup>3</sup>H) arginine monohydrochloride (64 Ci/mM) (Amersham, Arlington Heights, IL), 50 μL of 10 mM NADPH, and 50 μL of 6 mM CaCl<sub>2</sub>, (Calbiochem, CA) were added to each cell homogenate sample and incubated for 30 min at room temperature. After incubation, the reactions were stopped with 400 μL of stop buffer (50 mM HEPES, pH 5.5, 5 mM EDTA) and added with the equilibrated resin into each sample. The equilibrated resin bound unreacted arginine. After centrifugation, the radioactivity corresponding to L-(<sup>3</sup>H)-citrulline was measured with liquid scintillation spectrometry.

### 3.7. Determination of O<sub>2</sub><sup>-</sup> levels

O<sub>2</sub><sup>-</sup> production was determined spectrophotometrically (Hewlett Packard 8452A, CA) by monitoring the reduction of ferricytochrome c (Type VI, Sigma, St. Louis, MO) at 550 nm, as described by Pritchard (19). Ferricytochrome c (final concentration, 50 μM) was added directly to the cuvette containing the cells and Dulbecco's phosphate-buffered saline (DPBS) (final volume, 1 mL), in the presence or absence of superoxide dismutase [(SOD), 350 U/mL], and the changes in absorbance were followed for 10 min. The rates of O<sub>2</sub><sup>-</sup> production were calculated on the basis of the molar extinction coefficient of reduced ferricytochrome c [ $\epsilon = 21000 \text{ cm}^{-1}(\text{mol/L})^{-1}$ ]. Cell counts were used to calculate results as nanomoles of O<sub>2</sub><sup>-</sup> per 10<sup>6</sup> cells per minute.

### 3.8. Biochemical analysis

The concentrations of ADMA, SDMA, and L-arginine were determined by high-performance liquid chromatography (HPLC) as described previously (15). In brief, solid-phase extraction on polymeric cation-exchange

columns was performed after addition of monomethylarginine as the internal standard. After derivatization with ortho-phthalaldehyde reagent containing 3-mercaptopropionic acid, the analytes were separated by isocratic reverse-phase HPLC with fluorescence-based detection system. Laboratory parameters indicating liver and renal function, complete hematocytometer examination, and blood sugar levels were measured by routine methods in the clinical laboratory.

### 3.9. Echocardiographic Doppler evaluation

Echocardiography was performed using an ultrasound system (Vivid-e GE Healthcare Fairfield, Connecticut) with a 3.7-MHz transducer. LVEF was evaluated from apical 4- and 2-chamber views by using the Simpson's biplane method. Each representative value was obtained from the average of 3 measurements according to the American Society of Echocardiography criteria. The valvular assessment included evaluation of the functions of the mitral, aortic, and tricuspid valves. Color-Doppler echocardiography was performed after optimizing the gain and Nyquist limit, and standard continuous and pulsed-wave Doppler recordings were acquired. Stenotic and regurgitant valve diseases were evaluated according to the semiquantitative and quantitative methods recommended by the American Society of Echocardiography.

## 4. STATISTICAL ANALYSIS

Results are expressed as the mean ± standard deviation (SD) values. Data were analyzed using SPSS statistical software (version 15.0 for Windows; SPSS Inc., Chicago). For each baseline characteristic, the mean value or the corresponding percentage of study participants was calculated. The significance of changes in L-arginine, ADMA, and SDMA levels; eNOS expression and activity; and O<sub>2</sub><sup>-</sup> production was examined using the paired Student's t-test. A 2-tailed p value of <0.05 was considered significant.

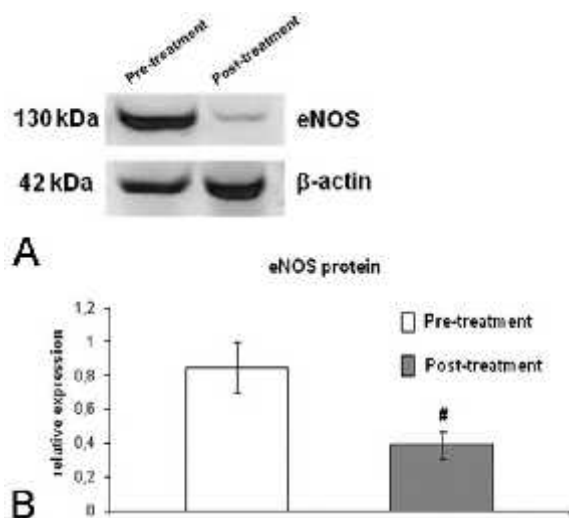
## 5. RESULTS

### 5.1. Baseline characteristics of the study population

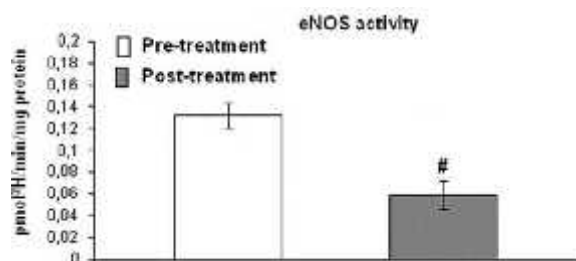
Twenty-four patients were included in the study. Baseline characteristics of the patients are summarized in Table 1. Thirteen patients were men, 13 patients were in NYHA functional class III, 11 patients were in NYHA functional class IV, and the mean age was 64 ± 6 years. Laboratory data (the mean ± SD values) are summarized in Table 2. There were no differences in the laboratory parameters indicating renal (creatinine, urea) or hepatic (AST, ALT) functions, or from a complete hemocytometer examination (hemoglobin, red and white blood cell counts, hematocrit) before and after therapy.

### 5.2. HPLC results

L-Arginine, ADMA, and SDMA were significantly higher in critically ill patients after pharmacological treatment (L-arginine, 1.78 ± 0.09 μM ; ADMA, 0.75 ± 0.26 μM; SDMA, 1.34 ± 0.37 μM) compared to basal (pre-treatment) levels (L-arginine, 1.33 ± 0.08; ADMA, 0.66 ± 0.22 μM; p < 0.01, SDMA 1.22 ± 0.35; p < 0.01) (Table 3)



**Figure 1.** The eNOS expression in PBMC of patients with diagnosis of acute congestive HF. A. Representative image of protein expression levels determined by Western blot analysis with antibodies against eNOS or  $\beta$ -actin as indicated on proteins extract from PBMC pre- and post-pharmacological treatment. The  $\beta$ -actin band intensities indicate equal loading of each well. B. The data shown (mean  $\pm$  S.D., n=24) indicate significant reduction of eNOS protein expression in PBMC of patients post-pharmacological treatment compared to pre-pharmacological treatment. The data were obtained from three independent experiments performed in triplicate (<sup>#</sup>P<0.05).



**Figure 2.** Endothelial nitric oxide synthase (eNOS) enzymatic activity in PBMC cells. The data show decreased levels of enzyme activities in patients with acute congestive HF after pharmacological treatment (mean  $\pm$  S.D., n=24) (<sup>#</sup>P<0.05). eNOS enzymatic activity was determined by measuring the L-[<sup>3</sup>H] arginine to L-[<sup>3</sup>H] citrulline, and are expressed in pmol <sup>3</sup>H min<sup>-1</sup> mg<sup>-1</sup> protein.

### 5.3. eNOS expression and activity

Western blotting was used to quantify eNOS protein levels in patients diagnosed with ACHF (Figure 1). It was found that treatment with pharmacologic agents (diuretics, digoxin, ACE inhibitors or angiotensin receptor blockers, and nitroglycerin) significantly decreased eNOS levels compared to the basal (pre-treatment) levels. L-[<sup>3</sup>H] citrulline production from L-[<sup>3</sup>H] arginine was detectable in the homogenates from each sample, and significant

differences in eNOS activity were observed between the samples (Figure 2).

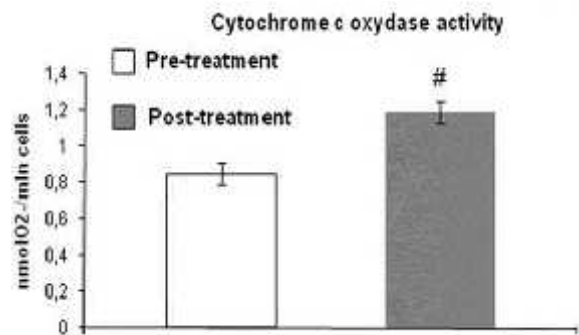
### 5.4. O<sub>2</sub><sup>-</sup> production

Since increased SDMA levels were already associated with a decrease in NO production and an increase in reactive oxygen species (ROS) production, we evaluated O<sub>2</sub><sup>-</sup> production in the PBMCs of patients after pharmacological treatment. O<sub>2</sub><sup>-</sup> production was determined spectrophotometrically by monitoring the reduction of ferricytochrome c. The data are shown in Figure 3.

## 6. DISCUSSION

The European Society of Cardiology (ESC) defines HF as a syndrome in which patients have the following symptoms: common shortness of breath at rest or during exertion, and/or fatigue; signs of fluid retention, such as pulmonary congestion or ankle swelling; and objective evidence of an abnormality in the structure or function of the heart at rest (20). CVD, as HF are one of the key causes of morbidity and mortality worldwide (21). There is abundant evidence that the endothelium plays a crucial role in the maintenance of vascular tone and structure. One of the major endothelium-derived vasoactive mediators is NO (11). Disturbances in NO bioavailability lead to altered regulation of key physiological and cellular processes, such as vasodilation, platelet function, and angiogenesis, so NO plays an important role in protection against the onset and progression of CVD (17). The synthesis of NO from L-arginine is catalyzed by NO synthase, a widespread biological mediator involved in many functions, including regulation of vascular tone, neurotransmission, and host defense (22). Various enzyme inhibitors block production of NO, such as L-NMMA and ADMA, which act as endogenous inhibitors of NOS.

Our observation of low plasma concentrations of dimethylarginine (DMAs) in healthy subjects is in good accordance with the literature (23-24). Although SDMA control values differ distinctly, the structural counterpart of ADMA has been considered inert. The role of SDMA in vascular dysfunction is not yet clear; however, since SDMA is an inhibitor of the human cationic amino acid transporter hCAT-2B, and it may indirectly inhibit NO synthesis by interfering with arginine uptake (25). A recent study showed that SDMA inhibits NO synthesis in cultured endothelial cells by competing with the transport of L-arginine, the substrate of NOS, and this effect was associated with an increase in ROS (26). In a meta-analysis by Kielstein *et al.*, SDMA was put forward as a possible marker of renal function. SDMA is a structural isomer of the endogenous NOS inhibitor ADMA. Although there is mounting evidence that chronically elevated ADMA levels may contribute to the progression of vascular diseases *via* endothelial damage, little attention has been paid to the role of SDMA. Both ADMA and SDMA derive from intranuclear methylation of L-arginine residues and are released into the cytoplasm after proteolysis. Until now, however, most studies in the field of dimethylarginines focused on the elevation of ADMA and SDMA levels during renal failure, and it is generally accepted that



**Figure 3.** O<sub>2</sub><sup>-</sup> production in PBMC of patients with acute congestive HF. O<sub>2</sub><sup>-</sup> production was normalized with respect to the cell number and is expressed as nmol O<sub>2</sub><sup>-</sup> per 10<sup>6</sup> cells min<sup>-1</sup>. O<sub>2</sub><sup>-</sup> production was significant increase in PBMC extracted from blood of patients post-pharmacological treatment respect to PBMC isolated from the same patients at the recovery time (pre-treatment). The data are shown as mean ± S.D (n=24) (#P<0.05).

accumulation of dimethylarginines is related to decreased capacity for renal elimination (27). SDMA does not interfere directly with NOS activity, as ADMA does, but it is a potent competitor of L-arginine transport. Since L-arginine is the amino acid from which NO is synthesized by eNOS, it has been proposed that L-arginine deficit contributes to impaired NO-dependent vasodilation (25).

In our study, analysis of the PBMCs from patients subjected to ordinary pharmacological treatments, compared to pre-treated samples, showed increased concentrations of L-arginine, SDMA, and ADMA. In addition, it was clearly evident that in the PBMCs from treated patients, the levels of eNOS protein were appreciably reduced compared to basal (pre-treatment) values. These results led us to believe that SDMA indirectly inhibits NO production by limiting the availability of arginine to eNOS. In fact, when these samples were analyzed for the metabolic activity of eNOS, the levels were unexpectedly reduced. Most likely, the deleterious effects of increased SDMA concentration are indirectly associated with increased ROS formation. Therefore, we determined the activity of cytochrome oxidase in PBMCs from pre- and post-treatment patients, and observed an increase in the metabolism of this enzyme, indicating elevated production of O<sub>2</sub><sup>-</sup> in treated patients. Under normal physiological conditions, O<sub>2</sub><sup>-</sup> is detoxified by the activity of superoxide dismutase (SOD), which converts it to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), and subsequently, water. However, if the O<sub>2</sub><sup>-</sup> level increases sufficiently, NO outcompetes SOD for O<sub>2</sub><sup>-</sup>, forming nitrates that are eliminated physiologically in the urine. The affinity of NO for O<sub>2</sub><sup>-</sup> is approximately 6-times faster than the dismutation of O<sub>2</sub><sup>-</sup> by SOD (28). This reaction displays a scavenging effect for NO (29). Since reduced bioavailability of arginine, presumably caused by the increase in plasma SDMA levels, reduces the fraction of active eNOS in the PBMCs of treated patients, the quantity of NO is not sufficient to act as a scavenger. This is an important result since it could explain the worsening condition of patients

pharmacologically treated for 2 weeks with diuretics, digoxin, ACE inhibitors or angiotensin receptor blockers, and nitroglycerin, where cardiac function diminished, and mortality increased. Our future objective is to clarify the mechanism by which pharmacological treatments influence the molecular network linking the nitroergic system with enzymes involved in the metabolism of DMAs, and to identify new potential therapeutic targets.

## 7. ACKNOWLEDGMENTS

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