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The biological effect of pharmacological treatment on dimethylaminohydrolases (DDAH-1) and cationic amino acid transporter-1 (CAT-1) expression in patients with acute congestive heart failure

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ABSTRACT

Aim: Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide (NO) which plays an important role in controlling vascular tone and regulates the contractile properties of cardiac myocytes. The aim of this study was to investigate the effect of pharmacological treatment on symmetric dimethylarginine (SDMA), ADMA and arginine plasma concentrations in patients with acute congestive heart failure (ACHF) through the evaluation of type-1 system cationic amino acid transporter-1/type 1 dimethylarginine dimethylaminohydrolases-1 (CAT-1/DDAH-1).

Methods and results: 25 hospitalized cardiology patients with symptomatic acute congestive HF (NYHA Class III-IV) and impaired left ventricular (LV) function (ejection fraction<35%) were included in the study. ADMA, SDMA, and arginine plasma concentrations were assessed before and after pharmacological treatment by high performance liquid chromatography. All patients received an adequate pharmacological treatment for ACHF. ADMA and SDMA plasma levels were significantly higher after pharmacological treatment respect to baseline values (pre-treatment) (0.75 vs 0.48; 1.31 vs 1.03; $p<0.01$). Arginine plasma concentration was significantly lower after therapy respect to baseline values (0.78 vs 0.99; $p<0.01$). This is associated more with the modulation of DDAH-1 protein than with of CAT-1 system transport.

Conclusions: In patients with ACHF, acute renal impairment function and the modulation of metabolism and extracellular transport by the DDAH-1/CAT-1 system determine high ADMA and SDMA levels after therapy for acute congestive heart failure.

Introduction

Symmetric dimethylarginine (SDMA) is the structural isomer of the endogenous nitric oxide synthase (NOS) inhibitor asymmetric dimethylarginine (ADMA). ADMA, has emerged as a novel cardiovascular (CV) risk factor in the setting of cardiovascular diseases (CVD) associated with endothelial dysfunction, including type 2 diabetes mellitus (Krzyzanowska et al., 2007), coronary artery disease (CAD) (Boger, 2003; Meinitzer et al., 2007), carotid atherosclerosis (Riccioni et al., 2010), and end stage renal disease (Zoccali et al., 2006). Systemic accumulation of ADMA has also been implicated in the pathogenesis of heart failure (HF) (Duckelmann et al., 2007). ADMA may be an important target for pharmacotherapeutic intervention (Boger, 2003). It is known that ADMA is synthesized by the protein arginine methyltransferases (PMRT), which utilizes an S-adenosyl-methionine methyl group donor. Type 1 PRMTs catalyze the formation of ADMA, whereas type 2 PRMTs lead to the formation of SDMA. ADMA is eliminated both by renal excretion and metabolic degradation, while the SDMA is only discharged by renal excretion. Its metabolism is facilitated by dimethylarginine dimethylaminohydrolases (DDAHs), which are expressed as type 1 and 2 isoforms: DDAH-1 and DDAH-2. DDAH-1 is the predominant isoform in the proximal tubules of the kidney and in the liver, but is also expressed strongly in the pancreas, forebrain, aorta, peritoneal neutrophils and macrophages (Palm et al., 2007). These organs extract ADMA from the circulation. DDAH-2 is the predominant isoform in the vasculature, where it is found in endothelial cells adjacent to the cell membrane and in intracellular vesicles and in vascular smooth muscle cells among the myofibrils and the nuclear envelope (Dayal et al., 2008).

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DDAH could have a pivotal role in maintaining the homeostatic integrity of the cardiovascular and renal systems. Accumulating data support a physiologic role for the nitric oxide (NO) signaling pathway in the regulation of cardiac inotropy and relaxation. The availability of the substrate L-arginine is fundamental for NO production (Mori and Gotoh, 2004). NO synthesized from L-arginine catalyzed by NO synthase is a wide spread biological mediator with many functions (Christopherson and Bredt, 1997). For example, NO plays an important role in the regulation of vascular tone, neurotransmission, and host defense (Kone, 1997). The dysregulation of NO metabolism as a contributing factor has been demonstrated in a number of vascular pathologies including atherosclerosis, hypercholesterolaemia, diabetes, hypertension and septic shock (Khalid, 2005). One of the major contributing factors to the progression of the cardiovascular disease is a loss of NO-dependent actions. It is proposed that this is due to a reduction in the bioavailability of NO. There are a variety of factors which could reduce NO availability including: reaction in the availability of the substrate L-arginine, increased concentration of circulating inhibitors such as ADMA (Vallance and Leiper, 2004). L-arginine is actively transported into cells via a specific transporter system for cationic amino acids (CATs) (Yeramian et al., 2006). Regulation of L-arginine transport is essential and abnormalities in regulation of this system can thus result in impaired NO production (Sobrevia and González, 2009). The critical step that determines the partition of ADMA and SDMA between the cytosol and the extracellular fluid is its transmembrane transport via CATs. CATs are widely distributed on cell membranes either as CAT-1 that transports methylarginine and arginine across cell membranes in blood vessels and the distal nephron of the kidney or as CAT-2A that transports these cationic amino acids (CAAs) across the membranes of liver cells (Baylis, 2006). Consequently PRMT, DDAH and CAT activities may all assume critical roles in determining cellular levels of ADMA. The synthesis of NO from its precursor L-arginine may be altered by the formation of a complex series of arginine methylation pathways in the presence of inflammation and oxidative stress through degradation of cellular proteins that contain arginine residue (Nicholson, et al., 2009). In particular, two isoforms of methylarginine have been identified as potent endogenous NOS inhibitors: *N*-mono-methylarginine (MMA) and its methylation product, ADMA (Vallance and Leiper, 2004).

Acute congestive heart failure (ACHF) is a syndrome manifesting as the inability of the heart to fill with or eject blood due to any structural or functional cardiac conditions (Hunt et al., 2005), responsible for more hospitalizations than all forms of cancer combined (Lloyd-Jones et al., 2009), which may be caused by myocyte death, myocyte dysfunction, ventricular remodeling or some combination (Hunt et al., 2009). Patients with HF were found to have increased circulating levels of ADMA when compared with healthy controls (Usui et al., 1998; Saitoh et al., 2003). Although elevated ADMA plasma concentrations have been described in patients with HF (Young et al., 2008) little is known about the plasma concentration of SDMA and arginine in patients with ACHF (Dückelmann et al., 2008). The aim of this study was to investigate the effect of pharmacological treatment on SDMA, ADMA and L-arginine plasma concentrations in patients with ACHF through the evaluation of system CAT-1/DDAH-1.

Materials and methods

Study design

Between March and July 2010 all consecutive patients with diagnosis of ACHF (defined as acute and progressive resting dyspnoea associated with clinical signs of pulmonary or peripheral congestion requiring hospitalization and treatment with an intravenous diuretic) admitted to the Intensive Cardiology Unit of San Camillo De Lellis Hospital (Manfredonia, Italy) were invited to participate in this study.

Eligible subjects were 50–75 years of age, with left ventricular ejection fraction (LVEF) ≤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 2× the upper limit of normal. Significant renal dysfunction was defined as estimated glomerular filtration rate (eGFR) ≤30 mL/min/1.73 m^2.

Patient population

Twenty-five patients with ACHF (LVEF<35%) were enrolled in the study. Medical and surgical history, physical condition, and medication were recorded. After inclusion, a heparinised blood sample was drawn from an indwelling arterial line for determination of ADMA, SDMA, and L-arginine at baseline. Subsequently a blood sample was drawn after one day of therapy. Simultaneously, laboratory parameters indicating renal function (creatinine, urea) and hepatic function (aspartate aminotransferase [AST], alanine aminotransferase [ALT]) and complete haematocytometer exam were determined before and after one day of therapy. The patients had been treated with diuretics, digoxin, angiotensin converting enzyme-inhibitors (ACE-I) or angiotensin receptor blockers (ARBs).

Sample collection, storage and preparation

Blood samples were collected in polypropylene tubes containing EDTA 1 mM. Samples were stored in an ice box prior to centrifugation at 3000 *g* for 10 min at 4 °C. 200 μl aliquots of plasma were transferred into an Eppendorf tubes. Plasma samples were either used for extraction immediately or stored in the dark at −80 °C until analysis was performed.

Isolation of human peripheral adherent mononuclear cells

Venous blood was collected by phlebotomy in EDTA vacutainers (6 mL K_2EDTA, Becton Dickinson, USA) and processed within 2 h of procurement. Serum was isolated from the blood. Primary blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation through Ficoll/Hypaque (Pharmacia). After centrifugation (200 *g*; 4 °C; ×25 min), the interphase layer containing (PBMC) was carefully removed, washed in phosphate buffered saline (PBS 1×) followed by centrifugation (600 *g*; ×15 min). The cell pellet was placed into lysis buffer [50 mmol L^{-1} Tris–HCl pH 7.5, 0.4% Nonidet P-40 (NP-40), 120 mmol L-1 NaCl, 1.5 mmol L-1 MgCl2, 2 mmol L-1 phenylmethylsulphonyl fluoride (PMSF), l lg mL-1 leupeptin, 3 mmol L-1 NaF and 1 mmol L-1 dithiothreitol] for 30 min at 4 °C. The protein concentrations of the extracts were determined using the Bradford method.

Biochemical analysis

The concentration of ADMA, SDMA and L-arginine were determined by high-performance liquid chromatography (HPLC) as described previously (Teerlink et al., 2002). In brief, solid-phase extraction on polymeric cation-exchange columns was performed after addition of monomethylarginine as the internal standard. After derivatization with ortho-phtaldialdehyde reagent containing 3-mercaptopropionic acid, analytes were separated by isocratic reversed-phase HPLC with fluorescence detection. Laboratory parameters indicating liver and renal function, complete haematocytometer exam, and blood sugar were measured by standard methods in the clinical laboratory.

Detection of DDAH 1 and CAT-1

Determination of DDAH-1 protein was performed in protein extracts by western blotting (WB). 50 μg cytoplasmatic, quantified by spectrophotometric assay (HP 8452A, Palo Alto, CA, USA) using the Bradford method, primary blood mononuclear cells were separated by electrophoresis in a 7.5% sodium dodecyl sulfate-polyacrylamide gel (SDS–PAGE; Bio-Rad, Hercules, CA, USA) and transferred at 4 °C to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) in glycine-methanol buffer. Nitrocellulose was then blocked in Tris-buffered saline (TBS)-milk and incubated overnight with various primary antibodies: anti-human DDAH-1 at dilution 1/1000 (Santa Cruz Biotechnology, CA, USA) and anti-human CAT-1 antibody at dilution 1/5000 (Santa Cruz Biotechnology, CA, USA). The nitrocelluloses were then washed in TBS, incubated with a secondary antibody conjugated with alkaline phosphatase for 2 h, washed again, and developed in an alkaline buffer with nitroblue tetrazolium (NBT) as substrate (Alkaline Phosphatase Conjugate Substrate Kit, Bio-Rad, Hercules, CA, USA). β-Actin (Sigma, 1/10000), was used as an internal standard. The resulting blot image files were imported and analyzed with commercially available gel analysis software package (Bio-Rad Gel Doc 1000, Milan, Italy).

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 four- and two-chamber views, using the Simpson's biplane method. Each representative value was obtained from the average of three measurements according to the American Society of Echocardiography criteria. The valvular assessment included the evaluation of the function of the mitral, aortic and tricuspid valves. Color-Doppler echocardiography was performed after optimizing gain and Nyquist limit, and standard continuous and pulsed-wave Doppler recordings were acquired. Stenotic and regurgitant valve diseases were evaluated according to semiquantitative and quantitative methods recommended by the American Society of Echocardiography. Tricuspid regurgitation was visualized from the apical 4-chamber view. The PAPs was estimated from the peak tricuspid regurgitation jet, using the simplified Bernoulli equation (PAPs = 4 × v2 + right atrial pressure), where "v" is the peak velocity of the tricuspid regurgitation jet (m/s), and the right atrial pressure is estimated from the diameter and breath-induced variability of the inferior vena cava. Data were stored for further off-line analysis.

Statistical analysis

Results were expressed as mean ± SD. Data were analyzed by using SPSS statistical software (version 15.0 for Windows; SPSS Inc., Chicago). For each baseline characteristic, the mean value or the corresponding percent of study participants was calculated. The significance of changes in ADMA, SDMA and L-arginine was examined using the paired Student's *t*-test. A two-tailed p value <0.05 was considered significant.

Results

Baseline characteristics of study population

Twenty-five patients were included in the study. Baseline patient characteristics are summarized in Table 1. Mean age was 64 ± 4 years, 15 patients were males and 10 females, 14 patients with III NYHA functional class and 11 patients with IV NYHA functional class.

Means ± SD of laboratory data are synthesized in Table 2. There was no difference in laboratory parameters indicating renal (creatinine, urea) and hepatic (AST, ALT) functions, and complete haematocytometer exam (hemoglobin, red and white cells, hematocrit) before and after therapy.

Table 1
Demographic details of population study.

Patient characteristics	
Age (mean ± SD)	64 ± 4
Sex (M/F)	15/10
BMI (Kg/m^2)	28.67 ± 3.43
NYHA functional class III	14
NYHA functional class IV	11

ADMA and SDMA plasma levels were significantly higher in critically ill patients after pharmacological treatment (ADMA 0.82 ± 0.12 μmol/L; SDMA 1.27 ± 0.13 μmol/L) compared to basal (pre-treatment) levels (ADMA 0.39 ± 0.15 μmol/L; $p<0.001$, SDMA 1.01 ± 0.11; $p<0.001$ respectively). Instead L-arginine plasma concentration was significantly lower after therapy with respect to baseline values (0.78 ± 0.10 vs 0.99 ± 0.11, $p<0.001$).

DDAH-1 protein expression

Western blot analysis of PBMC homogenates revealed the presence of an immune-reactive band of 31 Kda. Consistent over-expression of DDAH-1 protein is present in cells extract of patients after-pharmacological treatment (Fig. 1A). Densitometry revealed that DDAH-1 expression in PBMC was significantly lower in patients after-pharmacological treatment compared to pre-pharmacological treatment (Fig. 1B).

CAT-1 protein expression

We used Western blot analysis to measure levels of CAT-1 in PBMC isolated from the patients pre- and post-pharmacological treatment. We did detect a significant over-expression of CAT-1 in patients post-pharmacological treatment respect to baseline (Figs. 2A-B). So we have hypothesized that extracellular transport of methylated arginines is increased resulting in elevated plasma concentrations of ADMA and SDMA.

Discussion

The common pathophysiologic state of ACHF is extremely complex. Compensatory mechanisms exist on every level of organization from sub-cellular all the way through organ–organ interactions. Most important among these adaptations are the Frank–Starling mechanism (Krzyzanowska et al., 2007), in which an increased preload helps to sustain cardiac performance; alters myocyte regeneration and death (Meinitzer et al., 2007); myocardial hypertrophy with or without cardiac chamber dilatation, in which the mass of the contractile tissue is increased (Zoccali et al., 2006); and activation of neurohumoral systems (Duckelmann et al., 2007), especially the release of norepinephrine by adrenergic cardiac nerves, which augments myocardial contractility and includes activation of the renin-angiotensin-aldosterone system (RAAS), sympathetic nervous system (SNS), and other neurohumoral adjustments that act to maintain arterial pressure and perfusion of vital organs (Teerlink et al., 2002).

In ACHF, the finite adaptive mechanisms that may be adequate to maintain the overall contractile performance of the heart at relatively normal levels become maladaptive when trying to sustain adequate cardiac performance. As HF advances, there is a relative decline in the counterregulatory effects of endogenous vasodilators, including NO, prostaglandins (PGs), bradykinin (BK), atrial natriuretic peptide (ANP), and B-type natriuretic peptide (BNP). This occurs

Table 2
Laboratory data.

	Pre-treatment	Post-treatment	P-value
Renal function			
Creatinine (mg/dL)	1.82	1.69	ns
Urea (mg/dL)	87	84	ns
Hepatic function			
AST	29	28	ns
ALT	31	30	ns
Haematocytometer exam			
HGB L (g/d)	12.3	12.2	ns
Red cells	4.206.000	4.190.000	ns
White cells	8.420	8.655	ns
Hematocrit	35.55	36.15	ns
Left ventricular ejection fraction (%)	31	37	P<0.01
ADMA (μmol/L)	0.39 ± 0.15	0.82 ± 0.12	P<0.001
SDMA (μmol/L)	1.01 ± 0.11	1.27 ± 0.13	P<0.001
L-arginine (μmol/L)	0.99 ± 0.11	0.78 ± 0.10	P<0.001

ns: not significant.

simultaneously with the increase in vasoconstrictor substances from the RAAS and adrenergic systems (Onwuanyi and Taylor, 2007).

In our study we found significantly high levels of ADMA and SDMA after treatment of ACHF compared to basal (pre-treatment) values (Table 2). The reasons that can be explained are: (1) reduction of renal excretion of ADMA/SDMA due to acute renal impairment function; (2) increased methylation of proteins by the protein arginine methyltransferases (PMRT) and impaired metabolism of dimethylarginine dimethyilaminohydrolase (DDAH) due to shear stress that increases PRMT and DDAH expression/activity and stimulates the production of ADMA. However, the most common mechanism leading to accumulation of ADMA involves impaired metabolism of DDAH. The higher concentration of ADMA and SDMA demonstrates that in ACHF the metabolism of PMRT1 and PMRT2 are impaired (Rawal et al., 1995).

There are two pathways for the clearance of dimethylarginine from the cell: 1) ADMA is enzymatically degraded by DDAH, which cleaves ADMA into citrulline and dymethylamine; 2) ADMA and SDMA are exported from the cell to the plasma via CAT in the membrane. Likewise, clearance from the plasma compartment also occurs by two modalities: renal excretion and uptake by cells via CAT (Fleck, et al., 2003). The CAT activity is thus involved in both cellular release and uptake of ADMA, and such plays an important role in intracellular transport of ADMA (Teerlink et al., 2002). Therefore, CAT activity sustains a dynamic equilibrium of basic amino acids between cell and plasma (Smulders et al., 1997). Altered metabolism of ADMA and alterated biosynthesis of methylarginine could promote cellular export of ADMA and SDMA via CAT-1. We have found high levels of ADMA and SDMA compared to basal values (Table 2) due to up-regulation of CAT 1 system. Our results (Fig. 2) evidenced an over-expression of CAT-1 after drug-treatment causing an imbalance due to high capacity of the cell to release dimethylarginine by CAT.

This difference (pre and post treatment) in plasma ADMA levels may be related to the therapies used. The patients were treated with diuretics, digoxin, ACE-Is or ARBs. Several studies have assessed the effect of ACE inhibitors or ARBs on plasma ADMA levels. ACEI and ARB have been shown to decrease plasma ADMA in many studies (Delles et al., 2002; Ito et al., 2002). In patients with hypertension, diabetes or ipercholesterolemie, the reduction in plasma ADMA achieved by ACE inhibition was in the range of 12–20%, while plasma L-arginine levels did not seem to be affected (Chen et al., 2002). ARBs were found to reduce plasma ADMA by 10–16% (Delles et al., 2002; Ito et al., 2002). All of these studies were small and had rather short observation periods of 1–12 weeks. Thus, confirmation of these promising results by larger trials will be required. The results of a recent study in eNOS knockout mice suggests that ADMA may also exert NO-independent effects via upregulation of ACE and augmentation of oxidative stress through AT1-dependent pathways (Suda et al., 2004). Thus, ACE inhibitors and ARBs may help to prevent ADMA-induced pathology independently of their effects on plasma ADMA levels. In agreement with the results shown in this study, are the data from our previous work in which we evaluated the effect of pharmacological treatment on asymmetric dimethylarginine (ADMA) concentration in patients with acute congestive heart failure (Riccioni et al., 2011). Even if any of the drugs used improved endothelial function, only selective β-adrenergic blocker had a therapeutic benefit on endogenous NO production (Afonso et al., 2006). The precise mechanisms are yet to be defined, but there is data supporting the role of β-adrenergic blockers in preventing oxidative-stress induced up-regulation of PRMT

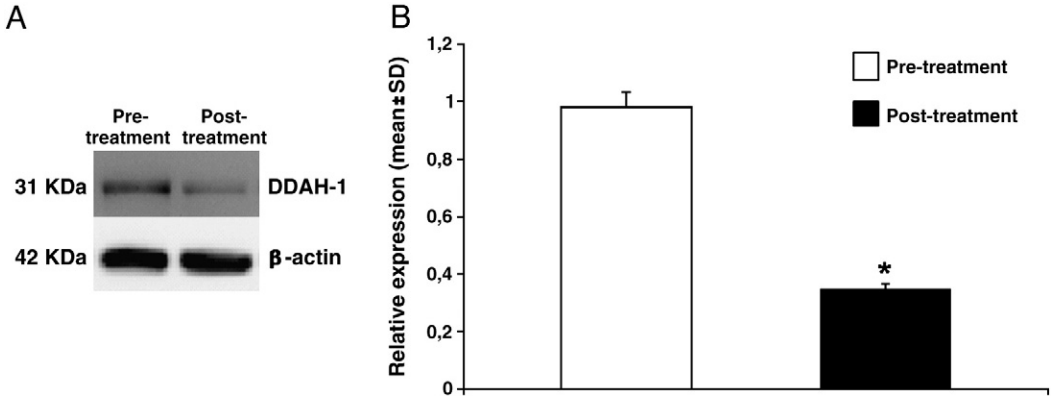

Fig. 1. DDAH expression in PBMC of patients with diagnosis of acute congestive heart failure. A. Representative image of protein expression levels determined by western blotting analysis with antibodies against DDAH-1 or β-actin on proteins extract from PBMC pre- and post- pharmacological treatment. The β-actin band intensities indicate equal loading of each well. B. Data shown (mean ± SD) indicate significant reduction of DDAH protein expression in PBMC of patients post-pharmacological treatment compared to pre-pharmacological treatment. All the data of the comparative studies had a p value (ANOVA<0.05).

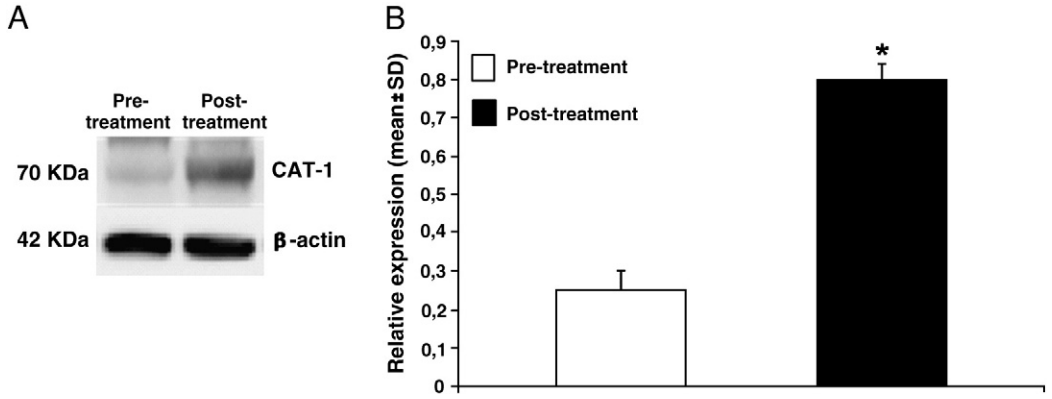

Fig. 2. Expression of the cationic amino acid transporter CAT-1 in PBMC of patients with diagnosis of acute congestive heart failure. A. Representative image of protein expression levels determined by western blotting analysis with antibodies against CAT-1 or β-actin on proteins extract from PBMC pre- and post- pharmacological treatment. The β-actin band intensities indicate equal loading of each well. B. Relative quantification of CAT-1 expression by densitometry. All the data of the comparative studies had a p value (ANOVA<0.05).

expression (Boger et al., 2000) or inhibition of DDAH activity (Ito et al., 2002). An alternative explanation can be the modulation of NOS expression (the target of ADMA antagonism) by β-adrenergic blockade. Our results show that drug-treatment in patients with acute congestive heart failure leads to an under-expression of DDAH-1 enzyme determining a lack of ADMA metabolism with consequent increase in its plasma concentration (Fig. 1). Moreover, the increased plasma concentration of ADMA could be a consequence of the reduced concentration of L-arginine, whose concentration is low enough not to compete with the ADMA for binding to CAT-1. Our findings show that pharmacological treatment with diuretics, digoxin, ACE-inhibitors or angiotensin receptor blockers interfere with plasmatic concentration of ADMA, SDMA and L-arginine, through modulation DDAH-1/CAT-1 system. Our study demonstrates that ACHF on ordinary pharmacological treatments have significantly higher ADMA levels when compared to pretreatment levels. Elevated plasma ADMA levels have been found in association with endothelial dysfunction in both animal and humans (Dayal et al., 2008; Zinellu et al., 2008). Since the ADMA is an endogenous NOS inhibitor and increased plasma ADMA levels are associated with cardiovascular morbidity, it is not completely understood how this happens and what will determine the long-term in these patients who have achieved an improvement after 24 h of clinical conditions. Our future aim will be to monitor as time goes by whether the ADMA levels related to a modulation of DDAH-1/CAT-1 system can lead to a relapse.

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