

BNP and iNOS in decompensated chronic heart failure: a linear correlation

Lorenza Speranza¹, Sara Franceschelli¹, Graziano Riccioni², Marta Di Nicola³, Benedetta Ruggeri⁴, Sabina Gallina^{1,4}, Mario Felaco¹, Alfredo Grilli¹

¹Department of Human Movement Sciences, University G. D'Annunzio, Chieti Italy, ²Cardiology Unit, San Camillo de Lellis Hospital, Manfredonia, Foggia, Italy, ³Department of Biomedical Sciences University G. D'Annunzio, Chieti Italy, ⁴Cardiology Department SS Annunziata Hospital, Via dei Vestini-Chieti Italy

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1. ABSTRACT

Heart Failure (HF) is characterized by activation inflammatory mediators that contributes to the disease progression. Brain natriuretic peptide (BNP) plasma levels increase in HF with a prognostic significance. The aim of this study was to evaluate the expression and activity of inducible nitric oxide synthase (iNOS) in peripheral blood mononuclear cell (PBMC) extracted from patients and a possible linear correlation between iNOS and plasma levels of BNP in decompensated chronic HF (DCHF) patients. To establish the DCHF, thirty-five male patients were evaluated. All patients were venesected within 24 h of admission to exclude an inflammatory state through evaluation of c-reactive protein. Only twenty subjects showed symptoms of DCHF were included in the study. Other patients were included in the control group. In DCHF, left ventricular ejection fraction (LVEF) % was reduced and systolic pulmonary artery pressure (PAPs) was increased. Furthermore, iNOS expression and BNP plasma levels were significantly higher in patients with DCHF as compared to controls group. These findings indicate that in DCHF patients, iNOS activity exhibits a significant linear correlation with plasmatic BNP level.

2. INTRODUCTION

The European Society of Cardiology, defines HF as a syndrome in which the patients should have the following symptoms: typical 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 of the structure or function of the heart at rest (1,2). The natriuretic peptides are a family of hormones that are involved in the regulation of blood pressure, electrolyte balance and fluid balance (3). Three types of natriuretic peptides have been identified: B-type natriuretic peptide (BNP), atrial natriuretic peptide (ANP), and C-type natriuretic peptide (CNP) (4). Release of ANP and BNP into plasma is stimulated by myocyte stretch, but endothelin-I, nitric oxide, and angiotensin II may all have a role. (5,6,7). Like many hormones, BNP is derived from preproBNP (134 amino acids), which is cleaved to proBNP (108 amino acids) and finally the N-terminal proBNP (NT-proBNP) peptide is removed, producing the active form of BNP which is 32 amino acids long. BNP has good sensitivity and specificity for detecting systolic left ventricular ejection fraction (LVEF <40% to 50%) or diastolic dysfunction. BNP or NT-proBNP levels are

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elevated in patients with DCHF, and several studies have shown the value of BNP or NT-proBNP for diagnosis of acute heart failure (8,9,10,11). In an acutely dyspnoic patient, low peptide levels (<100 pg/ml for BNP <300 pg/ml for NT-proBNP) rule out DCHF with a negative predictive value of >95%; very high peptide levels can achieve positive predictive values >95% (12). Nitric oxide (NO) is an ubiquitous signaling molecule involved in physiological and pathophysiological actions and it derives from a reaction catalyzed by NO synthase (NOS) (13). In the heart three NOS isoforms are expressed: endothelial NOS (eNOS) in endothelial cells and cardiomyocytes, neuronal NOS (nNOS) in sympathetic nerve terminals, and inducible NOS (iNOS) that produce higher NO levels than eNOS (14,15). Accumulating data support a physiologic role for nitric oxide signaling pathway in the regulation of cardiac inotropy and relaxation. Human study reported a positive relationship between iNOS gene expression and cardiac work in patients with advanced heart failure (16). Thus the functional significance of cardiac iNOS-mediated NO in human heart failure remains controversial. Literature reports that NO has a positive or negative inotropic effect, depending on the concentration and a role in DCHF (17). The natriuretic peptides (ANP, BNP, CNP) are often increased in patients affected by cardiac insufficiency, and the BNP is a marker of left ventricular asymptomatic dysfunction and of cardiac insufficiency (6). The aim of this study is to evaluate iNOS activation in peripheral blood mononuclear cell (PBMC) in patients affected by DCHF and the possible relationship between iNOS activity and plasmatic BNP levels.

3. PATIENTS AND METHODS

3.1 Study Population

The study population was composed of 35 male patients affected by heart valve diseases consecutively admitted to our Cardiology Department between January 2008 and May 2009. Admission was due to: the worsening of chronic heart failure; the need to establish a diagnosis in patients with recent onset of heart failure; indication and evaluation for haemodynamic study. At the time of recruitment, all patients underwent an initial evaluation that included history, physical examination, electrocardiogram (ECG), radiographic examination, and bi-dimensional echocardiography with color Doppler flow analysis. Written informed consent was obtained from all subjects. The experimental protocol was approved by the local Ethics Committee. DCHF was defined according to the European Society of Cardiology guidelines (18). Patients were also classified according to the New York Heart Association (NYHA) criteria, with I-II being mild symptoms and III-IV being moderate to severe symptoms (2). Exclusion criteria included acute coronary syndromes, diabetes mellitus, active infection, malignant or inflammatory diseases, chronic renal insufficiency, history of thromboembolism, the use of steroids, immunosuppressive and nitrate treatment. The C-reactive protein (CRP) serum levels was measured as a nonspecific marker for inflammation (Table 1). Current smokers and subjects who had smoked within 2 years were excluded from the study. For all patients therapy for heart failure

consisted of angiotensin-converting enzyme (ACE) inhibitors, Angiotensin II receptor Blocker (ARB), beta blockers (BB), diuretics, Warfarin, (Table 1). All hospitalized patients were venesected within 24 h of admission.

4. LABORATORY PROCEDURES

4.1. Natriuretic Peptide

BNP was measured in plasma samples (K_3EDTA) using the ADVIA Centaur System (Bayer Diagnostics, Tarrytown, New York, USA) following the manufacturer's specifications. The Bayer ADVIA Centaur BNP assay is a two-site (sandwich) immunochemiluminescent assay. The upper normal value for our laboratory is 100 ng/l.

4.2. 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 (20). 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 (21, 22).

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 ($PAPs = 4 \times v^2 + \text{right atrial pressure}$), 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 (23).

4.3. Cytosol preparation by PBMC

Venous blood was collected by phlebotomy in EDTA vacutainers (6mL K_2EDTA , Becton Dickinson, Franklin Lakes, NJ USA) and processed within 2 hours of procurement. Plasma was isolated from the blood. PBMC were isolated by density-gradient centrifugation through Ficoll/Hypaque (Pharmacia). After centrifugation (1500×rpm; 4°C; ×25 min), the interphase layer containing PBMC was carefully removed, washed in PBS (1×) followed by centrifugation (2500×rpm; ×15 min). The cell pellet was splitted into RIPA buffer [50 mM Tris-HCl pH 7.5, 0.4% Nonidet P-40 (NP-40), 120 mM NaCl, 1.5 mM $MgCl_2$, 2 mM phenylmethylsulphonyl fluoride (PMSF), 1 mg mL⁻¹ leupeptin, 3 mM NaF and 1 mM dithiothreitol], for western blot analysis and into homogenization buffer 1X (Calbiochem, San Diego, CA, USA) for activity evaluation. The protein concentrations of the extracts were determined using the Lowry method.

4.4. Citrulline synthesis

The measure of the conversion of L-arginine to L-citrulline (expressed as pmol $^3\text{H min}^{-1} \text{mg}^{-1}$ protein) is a standard assay method currently used to quantitative NOS activity. Briefly, 10 μL of radioactive arginine, L-(2,3,4,5- ^3H) Arginine Monohydrochloride 64 Ci/mM, 1 $\mu\text{Ci}/\mu\text{L}$ (Amersham, Arlington Heights, Illinois, USA) and 50 μL NADPH 10 mM (Calbiochem, San Diego, CA, USA) 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-(^3H)-citrulline was measured with liquid scintillation counter. Calcium was omitted from these incubations to favour the determination of the calcium-independent iNOS isoform

4.5. Measurement of nitrite levels

NO production by the cells was quantified by measuring the accumulation of nitrite in the plasma by the Griess reagent (0.25 M phosphoric acid, 30 mM sulphanilamide, 2 mM naphthyl-ethylene diamine). Nitrite levels were determined using a sodium nitrite standard curve and are expressed as $\mu\text{mol L}^{-1}$ per 10^6 cells as described by Shubhangi A. *et al* 2009 (24)

4.6. Western- Blot of iNOS and 3-nitrotyrosine proteins

Determination of iNOS protein and 3-nitrotyrosine (a stable end product of the ONOO $^-$ decomposition) was performed in protein extracts by western blotting (WB). 50 μg of cytoplasmatic proteins, were quantified by spectrophotometric assay (HP 8452A, Palo Alto, CA, USA) using the Lowry method, 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 iNOS at dilution 1/1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and monoclonal anti-3-nitrotyrosine antibody at a dilution 1/5000 (Santa Cruz Biotechnology, Santa Cruz, 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). Primary antibody anti- β -Actin (Sigma, 1/10,000), was used as an internal standard. The resulting blot image files were imported and analyzed with gel analysis software package (Bio-Rad Gel Doc 1000, Milan, Italy).

5. STATISTICAL ANALYSIS

The results were reported separately for the DCHF group and the control group. All qualitative variables were summarized as frequency and percentage and all quantitative variables as mean and standard deviation (SD).

Statistical significance of differences between groups for qualitative variables were assessed using the chi-squared test or Fisher's Exact Test, when appropriate. Non-parametric Mann-Whitney U test was applied for assessing the comparison of the quantitative variables between the two groups (DCHF group and control group). Spearman *rho* correlation coefficient was applied to evaluate the correlation between BNP and activity of iNOS. In all statistical tests the threshold of statistical significance is assumed to be equal to $p < 0.05$. Data were analysed by SPSS $^{\text{®}}$ Advanced Statistical $^{\text{TM}}$ 13 (2004, Chicago, IL, USA) and R open source software. (www.r-project.org)

6. RESULTS

6.1. The demographic, clinical and echocardiographic characteristics

Of the 35 patients hospitalized, 20 fulfilled the diagnostic criteria for decompensated chronic heart failure. The other 15 subjects were considered as control group, the characteristics are shown in Table 1. All patients with heart failure had documented reduction of left ventricular ejection.

6.2. Expression of iNOS and 3-nitrotyrosine proteins

To verify the presence of a high production of peroxynitrite, the expression of a protein that represents a biological marker of the nitrogen radicals was evaluate. In fact, the results of the analysis by Western blotting show significantly higher levels in the DCHF patients compared to the controls ($p < 0.001$) (Table 2). To verify whether the detected NO derived from iNOS, the Western blotting analysis of iNOS was carried out. Our data show that the level of iNOS was significantly higher in DCHF patients than in the control group, reflecting a higher production of NO. This experiment were conducted on 35 hospitalized patients and divided on the basis of diagnostic criteria in 20 DCHF and 15 control group. Data were reported as mean \pm standard deviation values as shows in Table 2.

6.3. NO production (activity of iNOS)

The iNOS activity, was increased in DCHF samples compared to that of control samples (1.2 ± 0.1 versus 0.7 ± 0.1 ; $p < 0.01$) (Table 2). This experiment were conducted on 35 hospitalized patients and divided on the basis of diagnostic criteria in 20 DCHF and 15 control group. Data were reported as mean \pm standard deviation values as shows in Table 2.

6.4. Nitrite levels

A number of methods exist for measuring NO in biological systems. One of these methods involves the use of the Griess diazotization reaction to spectrophotometrically detect nitrite formed by the spontaneous oxidation of NO under pathophysiological conditions. As shown in table II, the level of NO in the supernatants was significantly higher in the DCHF patients than in the control group. This experiment were conducted on 35 hospitalized patients and divided on the basis of diagnostic criteria in 20 DCHF and

Table 1. Clinical characteristics of control subjects and DCHF patients

Variable	Control group	DCHF group	p-value
Overall	15	20	
Age (years), mean±SD	71.5±8.2	68.6±6.3	0.310 ^a
NYHA functional class, n(%)			<0.001 ^b
1	9 (60.0)	2 (10.0)	
2	6 (40.0)	5 (25.0)	
3	-	9 (45.0)	
4	-	4 (20.0)	
Type of valvular diseases, n(%)			0.443 ^b
MR	8 (53.3)	9 (45.0)	
AoR	7 (46.7)	9 (45.0)	
AoS	-	2 (10.0)	
SBP (mmHg), mean±SD	125.0±21.0	115.0 ± 17.2	0.131 ^a
WBC(x10 ³ /μl), mean±SD	5.72±1.87	7.12±1.25	0.005 ^a
CRP(mg/dl), mean±SD	0.33 0.08	0.44 0.04	0.005 ^a
Cholesterol (mg/dl), mean±SD	176.1±28.1	141.3±32.5	0.002 ^a
Triglycerides (mg/dl), mean±SD	117.6±36.9	83.9±30.0	0.005 ^a
Hb (g/l), mean±SD	13.0±1.4	11.6±1.4	0.006 ^a
Creatinine (mg/dl), mean±SD	1.2±0.5	1.5±0.7	0.168 ^a
Glycaemia (mg/dl), mean±SD	110.0±47.2	102.7±34.0	0.598 ^a
Obesity (BMI), mean±SD	9 (66.6)	6 (30.0)	0.097 ^b
Comorbidity, n(%)	11 (73.3)	20 (100.0)	0.026 ^b
Treatment, n(%)			
Acel	12 (80.0)	12 (60.0)	0.281 ^b
ARBs	4 (26.7)	4 (20.0)	0.700 ^b
BB	9 (60.0)	8 (40.0)	0.315 ^b
Diuretic	11 (73.3)	14 (70.0)	0.999 ^b
Warfarin	5 (33.3)	12 (60.0)	0.176 ^b

^aMann-Whitney U test DCHF vs Control group; ^bChi-squared test or Fisher exact test when appropriate, DCHF vs Control group
SBP=Systolic Blood Pressure; MR=Mitral Regurgitation; AoR=Aortic Regurgitation; AoS=Aortic Stenosis; HB=Hemoglobin; Ace I=ACE inhibitors; ARBs=Angiotensin II Receptor Blockers ; BB= beta-adrenergic receptor blockers. The definition for obesity is having a BMI greater than or equal to 30 kg/m² (19)

Table 2. Values of EF%, PAPs, BNP level, iNOS expression and activity, 3-nitrotyrosine expression, nitrite level

Variable	Control group (n=15)	DCHF group (n=20)	p-value ^a
EF (%)	61.1±2.4	37.2±10.1	<0.001
PAPs (mmHg)	19.7±3.4	40.0±5.5	<0.001
BNP (ng/L)	26.7±3.3	239.1±88.9	<0.001
iNOS protein (OD)	1.1±0.2	2.4±0.5	<0.001
iNOS (pmol 3H min ⁻¹ mg protein)	0.7±0.1	1.2±0.1	<0.001
Nitrotyrosine protein (OD)	1.1±0.2	2.8±0.5	<0.001
Nitrite Level (μmol L ⁻¹ /10 ⁶ cells)	3.1±1.0	9.1±1.4	<0.001

Mean ± standard deviation values of EF%, PAPs, BNP level, iNOS expression and activity, 3-nitrotyrosine expression, nitrite level in two groups (control and DCHF); BNP was assayed in plasma, iNOS protein, iNOS activity, and 3-nitrotyrosine were assayed in PBMCs and that nitrite was assayed in plasma., ^a Mann-Whitney U test DCHF vs Control Group, EF= Ejection Fraction; PAPs= Pulmonary Systolic Pressure; BNP= B-type Natriuretic Peptide; iNOS= inducible Nitric Oxide Synthase

15 control group. Data were reported as mean ± standard deviation values as shows in Table 2.

6.5. Natriuretic peptide

Significant differences were evident between groups. BNP plasma levels increased in patients with decompensated heart failure (39.1 ng/l). Patients without heart involvement had significantly lower BNP levels (26.7 ng/l, p< 0.001; Mann Whitney U-test) (Table 2). The appropriate cut-off value for the diagnosis of heart involvement was 100 ng/l. With this cut-off, sensitivity was 98% and specificity was 79%. In the patients with DCHF, as expected, there was increased BNP compared to the control group (239.1±88.9 versus 26.7±3.3; p<0.001), lower EF% (37.2±10.1 versus 61.1±2.4; p<0.001), and higher pulmonary systolic pressure values (40.0±5.5 versus 19.7±3.4; p<0.001) compared to the control group (Table 2). This experiment were conducted on 35 hospitalized patients and divided on the basis of diagnostic criteria in 20 DCHF and 15 control group. Data were reported as mean ±

standard deviation values as shows in Table 2.

6.6. Correlations between BNP level and iNOS activity

The analysis carried out demonstrate, for the first time, a statistically significant correlation between the BNP levels and iNOS activity. In the DCHF group we found that iNOS expression was positively correlated with BNP values (rho=0.670; p<0.001), while in the control group no correlation was found between iNOS expression and BNP value (rho=-0.003; p=0.992) (Figure 1). The overall subject correlation between iNOS and BNP was rho=0.861 (p<0.001).

7. DISCUSSION

Clinical investigations of natriuretic peptides have focused on the diagnostic usefulness for HF and left ventricular dysfunction and their prognostic usefulness in chronic HF, acute coronary syndromes, stable coronary artery disease, and other medical conditions (25). We

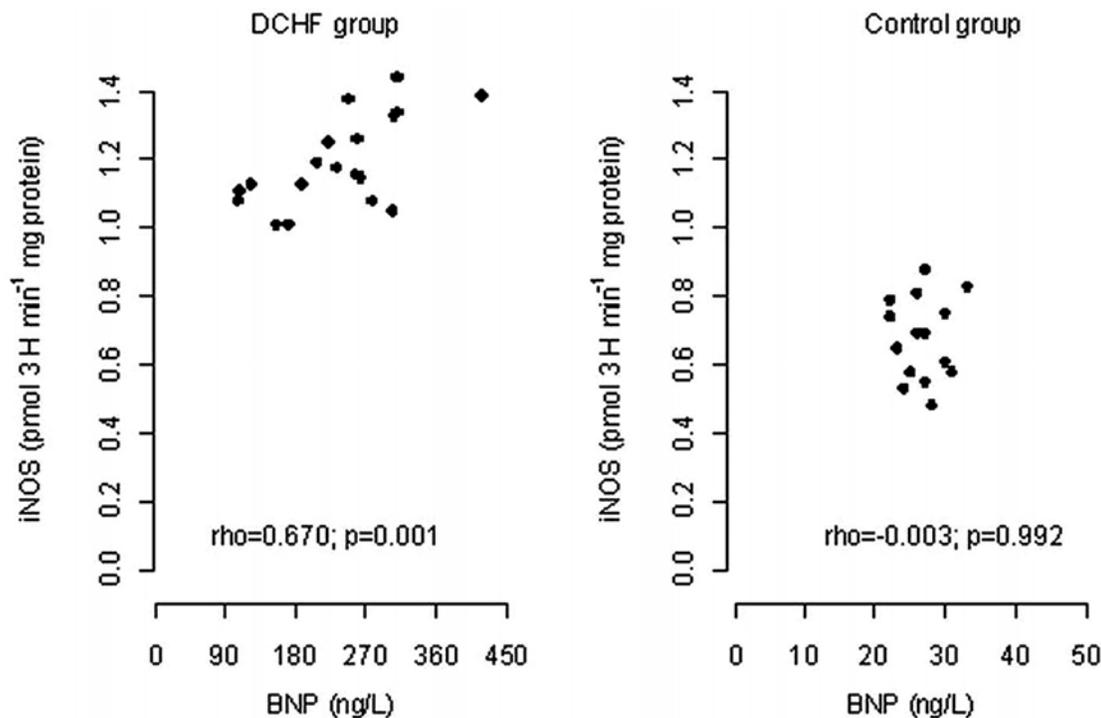


Figure 1. Scatterplot between plasma BNP levels and iNOS activities in the DCHF and control patients. There is a significant correlation between BNP levels and iNOS activities in the DCHF patients compared to a control group where such a correlation does not exist ($\rho=0.670$; $p=0.001$ vs $\rho=0.003$; $p=0.992$).

found, in accordance with the literature, that plasma natriuretic peptide levels are increased in patients affected by DCHF compared to the control group (Table 2). In our study we used BNP values not only to improve the diagnostic accuracy of DCHF, but also to evaluate the severity of haemodynamic compromise. In fact, the relation of BNP concentration to HF severity has also been noted by analytical parameters evaluated by echocardiography and nuclear imaging. Our data show a linear correlation between iNOS activity and BNP values (Figure 1). It is well-known that iNOS is either undetectable or detected at only low levels in healthy human hearts (26,27). The pathophysiologic mechanisms underlying HF progression are numerous. One hypothesis proposes that excessive nitric oxide (NO) production contributes to HF progression and LV dysfunction (28). Recent studies have shown that iNOS is expressed at high levels in the myocardium of failing hearts (29,30). In DCHF there is an increase of iNOS expression as demonstrated by our results (Table 2). Experimental studies have shown that high levels of NO inhibit the cardiomyocyte-shortening velocity, in part by activating guanylate cyclase and increasing cyclic guanosine monophosphate (31). In the plasma of patients affected by DCHF, the quantity of pro-inflammatory cytokines is high because of the activation of the neurohormonal and immune systems. Many studies have demonstrated how inflammation causes an oxidative stress which leads to the activation of pro-inflammatory signal transduction, such as NF- κ B or MAP kinases that activate different enzymes, such as iNOS, which produce high

amounts of NO (32,33). Oxidative stress has long been implicated in clinical and experimental HF (34). The term refers to an imbalance between the production of reactive oxygen species (ROS) including free radicals (such as superoxide and non-radicals such as hydrogen peroxide) and endogenous antioxidant defence mechanisms. ROS may in fact exert multiple effects relevant to HF pathophysiology (35). The discovery that mammalian cells have the ability to synthesize the free radical NO has stimulated an extraordinary impetus for scientific research in all the fields of biology and medicine. Since its early description as an endothelial-derived relaxing factor, NO has emerged as a fundamental signaling device regulating virtually every critical cellular function, as well as a potent mediator of cellular damage in a wide range of conditions (36).

Recent evidence indicates that most of the cytotoxicity attributed to NO is due more to peroxynitrite (ONOO⁻), produced from the diffusion-controlled reaction between NO and another free radical such as the superoxide anion (O₂⁻) (44). ONOO⁻ interacts with lipids, DNA, and proteins via direct oxidative reactions or via indirect, radical-mediated mechanisms. The hypothesis that ONOO⁻ generation contributes to myocardial and vascular dysfunction during ischemia and reperfusion (I/R), myocarditis, chronic heart failure, and various other cardiovascular pathologies has been the focus of intensive investigations during the last decade (37). Based on our results, since an increased expression of iNOS is significant

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in the decompensated patients in proportion to its metabolic activity, (Table 2), the production of NO is extremely high, as shown by the levels of nitrite. The role of NO is particularly important because, when produced by the activation of the guanylate cyclase (sGC9-cGMP) as signal transduction pathway, it mediates various physiological/beneficial effects in the cardiovascular system including vasodilatation, inhibition of platelet aggregation, anti-remodeling and anti-apoptotic effects (31). But, under pathological conditions associated with increased oxidative stress and inflammation, such as patients with DCHF, NO and superoxide O_2^- react to form ONOO $^-$, as noticeable from the 3-nitrotyrosine expression in the PBMC of the decompensated patients compared to the control group (Table 2). HF syndrome is complex and is frequently associated with tissue hypoperfusion, leading to hypoxia, which in turn may increase oxidative stress and inflammation. Moreover, we found a correlation between iNOS increase and hemodynamic overload expressed by BNP values in patients with decompensated chronic heart failure whereas the correlation was absent in the control group. We have demonstrated for the first time a significant correlation between BNP levels and iNOS activity in decompensated patients compared to a control group where such a correlation does not exist; we therefore hypothesize that iNOS could be considered as a marker of hemodynamic compensation (Figure 1). This evidence could be important since it may represent a new marker of hemodynamic overload that emerges before many other clinical markers of impaired left ventricular function, such as low ejection. Moreover, we found a correlation between iNOS increase and the entity of hemodynamic overload in patients with DCHF compared to controls. Our preliminary results are very interesting because, until now, iNOS was always correlated only to indirect left ventricular function parameters, as the ejection fraction. In fact, in a recent study the authors demonstrated, in patients with end-stage heart failure who had undergone ventricular assistance device or heart transplantation, that mechanical unloading of the heart normalizes myocardial iNOS expression in direct association with a reduction in cardiomyocyte apoptosis (38,39).

In the future we believe that the possible development of rapid, accurate and affordable diagnostic methods will allow the assessment and routine monitoring of BNP and iNOS in a day-to-day clinical setting, as well as in clinical trials.

8. ACKNOWLEDGMENTS

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23. Authors/Task Force Members, N. Galiè , M.M. Hoeper , M. Humbert , A. Torbicki , Vachieri JL, J.A. Barbera , M. Beghetti , P. Corris , S. Gaine , J.S. Gibbs , M.A. Gomez-Sanchez , G. Jondeau , W. Klepetko , C. Opitz , A. Peacock , L. Rubin , M. Zellweger , G. Simonneau ; ESC Committee for Practice Guidelines (CPG), A. Vahanian , A. Auricchio , J.Bax , C. Ceconi , V. Dean , G. Filippatos , C. Funck-Brentano , R. Hobbs , P. Kearney, T. McDonagh, K. McGregor , B.A. Popescu , Z. Reiner, U. Sechtem , P.A. Sirnes , M. Tendera , P. Vardas, P. Widimsky; Document Reviewers, U. Sechtem , N .Al Attar , F. Andreotti , M. Aschermann , R. Asteggiano , R. Benza , R .Berger, D. Bonnet , M. Delcroix , L. Howard , A.N. Kitsiou , I. Lang, A. Maggioni , J.E. Nielsen-Kudsk , M. Park , P. Perrone-Filardi, S. Price , M.T. Domenech , A. Vonk-Noordegraaf , J.L. Zamorano . Guidelines for the diagnosis and treatment of pulmonary hypertension, The Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS), endorsed by the International Society of Heart and Lung Transplantation (ISHLT). *Eur Heart J*; 20, 2493-537 (2009)

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Send correspondence to: Lorenza Speranza, Dept. of Human Movement Sciences, University G. D'Annunzio, Via Dei Vestini, 31, 66123 Chieti Italy, Tel: 39871-3554550, Fax: 39871-3554551, E-mail: l.speranza@unich.it

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