Free Radical Research, April 2011; 45(4): 379–388

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Manganese superoxide dismutase dimorphism relationship with severity and prognosis in cardiogenic shock due to dilated cardiomyopathy

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(Received date: 21 May 2010; Accepted date: 5 October 2010)

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Abstract

The aim was to determine (a) Ala-16Val-SOD2 dimorphisms; (b) allelic frequency and phenotype of a common Pro-Leu polymorphism in GPx1, in a cohort of patients with a cardiogenic shock (CS) due to dilated cardiomyopathy without acute coronary syndrome. Consecutive patients with *de novo* CS that worsened a dilated (DCM) or ischemic (ICM) cardiomyopathy. Congenital heart disease, pacemaker and other shock aetiologies were excluded. To determine oxidative stress (OS), this study evaluated lipid peroxidation, protein oxidation and erythrocyte GPx, SOD and catalase activities. Ala16Val-SOD2 (dbSNP: rs4880) and Pro198Leu-GPx1 (dbSNP: rs1050450) polymorphisms were studied by allelic discrimination using fluorogenic probes and the 5'nuclease (TaqMan) assay. Twenty-four patients (with ICM (n = 8) or DCM (n = 16), age = 57.5 ± 10.7 years, LVEF = 25.3 ± 8.5%, NT-proBNP levels = 8540 ± 1703ng/L) were included during a 15 month follow-up. OS parameters were significantly higher in patients than in controls. Distribution of MnSOD genotypes was 47% Val/Val-variant, 29.5% Ala/Val and 23.5% Ala/Ala-variants. Severity of CS was more important in patients with Val/Val-variant and can be put in parallel with NT-proBNP levels (Val/Val-variant: 11 310 ± 3875 ng/L vs Ala/Ala-variant: 6486 ± 1375 ng/L and Ala/Val-variant: 6004 ± 2228 ng/L; $p < 0.05$) and hemodynamic support duration (144.6 vs Ala/Val-variant: 108.8 h and Ala/Ala-variant: 52.5 h; $p < 0.05$) with a positive correlation (Spearman rho = 0.72, $p < 0.05$). Moreover, Val/Val-variant significantly influenced the mortality (Spearman rho = 0.67, $p < 0.05$), but not the morbidity ($p = 0.3$). Distribution of GPx genotypes was 64% Pro/Pro, 18% Pro/Leu and 18% Leu/Leu. GPx-variants influenced neither GPx activities nor cardiac events. In conclusion, CS was associated with markers of increased OS. GPx polymorphism did not influence the GPx activity. Only the Val-encoding MnSOD allele was significantly correlated with the severity and prognosis of CS.

Keywords: *Cardiogenic shock, oxidative stress, MnSOD polymorphism, GPx allele*

Abbreviations: *CS, Cardiogenic shock; ROS, Reactive oxygen species; OS, Oxidative stress; ICM, Ischemic cardiomyopathy; DCM, Dilated cardiomyopathy; ACS: Acute coronary syndrome; LVEF: Left ventricular ejection fraction; LVFS: Left ventricular fractional shortening; NT-proBNP, Amino terminal pro-brain natriuretic peptide; MDA: Malondialdehyde; oxLDL: Oxidized low density lipoprotein; CP: Carbonylated protein; MnSOD: Superoxide dismutase 2; GPx: Glutathione peroxidase.*

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2011 Informa UK, Ltd.
DOI: 10.3109/10715762.2010.532792

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Introduction

Oxygen radical-mediated tissue damage has been involved in a large number of pathological conditions including cardiovascular diseases [1–3], neurological disorders [4,5], cancer and the ageing process. Substantial evidence of increased myocardial OS and reactive oxygen species (ROS) have been observed in animal models of heart failure (HF) and involved in the pathogenesis of cardiac injury and progression of HF clinical syndrome. Moreover, increases in OS generally cause reactive gains in the expression of antioxidant defences [6]. Excessive free-radical generation may arise from many sources, including NAD(P)H oxidase, xanthine oxidases, auto-oxidation of catecholamines, nitric oxide synthase activation or mitochondrial leakage [7]. Under physiological circumstances, the majority of oxygen radicals is generated into mitochondria as by-products of electron transport and oxidative phosphorylation required for the production of ATP [8].

Cells are protected against metabolic OS by several enzymatic and non-enzymatic defence systems, including superoxide dismutase (SOD), glutathione peroxidase (GPx) and reduced glutathione [9]. Three isoforms of SOD are present: Cu,Zn-SOD (SOD1 gene, cytosolic protein), Mn-SOD (SOD2 gene, mitochondrial protein) and EC-SOD (SOD3 gene, extracellular SOD) [10]. The major extracellular scavenger of superoxide anions is EC-SOD mainly located in the extracellular space (90%) and is more involved in endothelial dysfunction, but major modifications due to HF more often imply mitochondrial changes [11,12]. Several studies have examined the role of Cu, Zn-SOD on atherogenesis and endothelial function but failed to observe protective effects under pathological conditions, such as reperfusion injury [13,14]. Manganese superoxide dismutase (MnSOD) has been the subject of particular interest as it is located in mitochondria and can be induced by several cytokines and by superoxide anion; it also appears to be involved in other processes, including tumour suppression and cellular differentiation [15]. A number of polymorphisms in this sequence have been described, but only Ala16Val has been demonstrated to have a functional significance [16].

In HF, some mitochondrial changes are known: they include a decrease of mitochondria number, an enhanced OS due to high levels of free fatty acids and increased tissue hypoxia [17]. Genetic impairment was revealed by idiopathic cardiomyopathy when using polymerase chain reaction (PCR) gene amplification. Mitochondrial DNA deletion was located in ATPase 6 gene and in the D-loop region [18]. Thus, these mitochondrial DNA mutations could be an important contributing factor to cardiomyopathy (CM). Some single nucleotide polymorphisms in GPx and SOD alter their function and increase the risk of disease such as cardiovascular disease or atherosclerosis, cancer, macular degeneration and cochlear damage [19–21]. In cardiogenic shock (CS), we hypothesized that one of the principal functions of MnSOD was to protect mitochondrial enzymes by direct inactivation of superoxide anion, a product of electron transport and oxidative phosphorylation. Moreover, a proper balance is required between activities of MnSOD and GPx1 (i.e. the cytosolic and mitochondrial isoform of GPx) enzymatic activities to protect mitochondria against the intermediate product hydrogen peroxide.

The aim of this study was to determine the allelic frequency and phenotype of common polymorphisms of MnSOD and GPx1 in a population with CS due to severe cardiomyopathy (CM) without acute coronary syndrome (ACS). We analyzed (a) common Pro/Leu polymorphisms in GPx1; (b) Ala-16Val-SOD2 dimorphisms (*Ala* or *Val* variant); and (c) the involvement in CM.

Materials and methods

Patients

Between August 2006 and March 2007, 100 patients with severe heart failure (HF) were recruited and included 24 patients with a *de novo* CS. All of these 24 consecutive patients had a 30-month follow-up.

Inclusion criterion was a *de novo* CS due to cardiomyopathy (CM) correlated with severe left ventricular (LV) dysfunction but without acute coronary syndrome (ACS). CS was documented by the presence of: (a) a dyspnea NYHA IV class combined with impaired end-organ perfusion, clinical signs of cold skin, decreased mental performance or oliguria and a systolic BP below 90 mmHg despite adequate inotrope treatments; (b) an underlying heart disease with systolic LV dysfunction; (c) a characteristic chest X-ray showing cardiomegaly, venous congestion and pulmonary oedema. Patients with ACS, HF with preserved systolic function, pacemaker, dialysis, liver disease, malignancy, familial cardiomyopathy, those with CS caused by congenital heart disease and other shock aetiologies such as sepsis, anaphylaxis, haemorrhage or hypovolemia and patients receiving treatments that change OS status (calcium antagonists or thiol-containing agents like captopril) were excluded.

Initial evaluation included history, clinical examination, ECG, chest X ray with measurement of cardiothoracic ratio, biological evaluations and echocardiography, performed at admission. Medications used before, during and at the time of hospital discharge were recorded.

Upon admission, an echocardiography, using an iE53 Philips machine equipped with a 2.5 MHz probe

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and TDI was performed, in accordance with recommendations of the American Society of Echocardiography. Severe systolic LV dysfunction was defined by a LV ejection fraction (LVEF) $< 35\%$ and/or a LV fractional shortening (LVFS) $< 25\%$. Evaluation of end-diastolic pressure was performed with Pulsed Doppler analysis and Pulsed wave TDI of the mitral annulus. The E/Ea index was calculated and an elevated end-diastolic pressure was defined with an E/Ea > 12.

Patients had a coronary angiography with left ventriculography and measurements of LV end diastolic pressure. Ischemic heart disease was defined when angiographic evidence of $\geq 50\%$ occlusion or ≥ 1 major coronary artery or previously confirmed myocardial infarction according to the World Health Organization criteria for symptoms, enzyme elevation or electrocardiographic changes with segmental wall motion abnormalities on echocardiography. Patients were divided into two groups: (a) ischemic cardiomyopathy (ICM) associating CM with coronary arteries stenosis and (b) dilated cardiomyopathy (DCM) associating CM with normal coronary arteries. For patients with DCM, endomyocardial biopsy (EMB) was not performed following reports on the role of EMB in the management of cardiovascular disease [22].

The study was approved by our institutional board and written informed consent was obtained from all patients.

Biological parameters. Upon admission, four samples of non-fasting blood were obtained by venipuncture and collected into heparinized or EDTA containing tubes and into tubes without any anti-coagulant (Vacutainer®, Becton-Dickinson, Rungis, France), centrifuged at 4500 rpm and 4°C for 10 min. Plasma and buffy coats were removed and erythrocytes were washed three times with 0.15 M NaCl solution. Aliquots of washed erythrocytes and plasma were stored at −80°C until analysis. For erythrocyte assays, the erythrocytes were subjected to haemolysis by thawing and diluted (1:10) in 10 mM phosphate buffer pH 7.8.

We performed several consecutive measurements, i.e. upon admission, at inotrope treatment withdrawal (dobutamine) and 1 month after cardiac stabilization with medical treatment including diuretics and/or anti-aldosterone, ACEi and before β-blockers introduction (results not shown).

Cardiac biomarkers. A Vitros ECI system was used for the chemiluminescent measurement of Amino Terminal-pro-B-type Natriuretic Peptide (NTproBNP) (Ortho Clinical Diagnostics, Issy-les-Moulineaux, France). A systematic determination of creatine kinase activity and troponin I concentrations was performed (Vitros 950 and Vitros ECI system).

Other biochemical parameters. Plasma glucose levels were measured by glucose oxidase method (Bayer Diagnostics, Puteaux, France). HbA1c levels were measured with ion-exchange high performance liquid chromatography (HPLC) normalized in accordance with the 'Diabetes Control and Complication trial (DCCT)' reference method improved with a CV $<$ 4% (Bio-Rad, Hercules, CA). Serum lipid levels (triglycerides, total cholesterol, HDL- and LDL-cholesterol) were measured by automated enzymatic methods (Randox, Crumlin, UK); serum creatinine by automated Jaffé method (Bayer Diagnostics, Puteaux, France). Inflammatory status was assessed by CRP and fibrinogen measurements.

Oxidative stress parameters. OS parameters were measured as previously described [23]. Plasma and erythrocyte lipid peroxidation was assayed using an improved fluorimetric method (Shimatsu RF-540 spectrofluorimeter) to determine malondialdehyde (MDA) [24] and oxidized low density lipoproteins (oxLDL) were assayed with a 'sandwich direct' technique using an Elisa commercial kit (Mercodia, Sweden). Assays of carbonylated proteins (CP) were done with a sensitive colourimetric immunoassay using a commercial kit of Elisa Zentech (Zenith Technology, New Zealand) after derivatization with dinitrophenylhydrazine (DNPH) and use of anti-DNPH-antibody. CP levels allowed evaluating protein oxidation.

Activity of erythrocyte Cu/Zn SOD was assayed on a multiparametric analyser CX4 (Beckman), using a commercial kit (Ransod®, Randox, UK) based on the production of $O2^{\bullet -}$ anions by the xanthine/xanthine oxidase system and the further reaction of these free radicals with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-(phenyltetrazolium) chloride to result in a red formazan (absorption wavelength = 505 nm). Erythrocyte GPx activity was determined with a commercial kit (Ransel®, Randox, UK) using a method based on the oxidation of reduced glutathione in the presence of cumene hydroperoxide, as developed by Paglia and Valentine [25]. Catalase activity was determined in erythrocytes using a spectrophotometric method developed by Johansson and Borg [26].

Haemoglobin (Hb) concentration was determined in the 1:10 diluted haemolysate by the cyanmethemoglobin method, using Sigma Diagnostic reagents (Sigma, Saint Quentin Fallavier, France). Specific erythrocyte enzyme activities were expressed in U/g Hb.

Erythrocyte-reduced glutathione (GSH) and glutathione disulphide (GSSG) were separated and quantified simultaneously by reverse-phase HPLC

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(Dionex, France) and coulometric detection. Usual values for oxidative stress parameters were obtained in 100 healthy subjects aged 20–45 years, without cardiovascular risk factors (diabetes, smoking, hypertension, dyslipidemia).

Marker selection. Genomic DNA was extracted from each patient's mononuclear cells in peripheral blood using a MagNA Pure Compact Instrument with a magnetic-bead technology for the isolation process according to manufacturer's instructions (Roche Diagnostics, Meylan, France)

We genotyped the Ala16Val-SOD2 (dbSNP: rs 4880), Pro198Leu-GPx1 (dbSNP: rs 1050450) polymorphisms by allelic discrimination using fluorogenic probes and the 5' nuclease (TaqMan) assay. The Ala-16Val-SOD2 and Pro198Leu-GPx1 polymorphisms were genotyped using the TaqMan SNP genotyping products: C-8709053-10 and 002-1770-CT, respectively (Applied Biosystems, Foster City, CA).

PCR reactions consisted of 1X TaqMan Universal PCR master mix (Applied Biosystems), 1X assay mix and 20 ng DNA. Real-time PCR was performed on a Step One Plus PCR system (Applied Biosystems) using a protocol consisting of incubation at 50°C for 2 min and 95°C for 10 mn, followed by 40 cycles, denaturation at 92°C for 15 s and annealing/extension at 60°C for 1 min. The FAM and VIC fluorescence levels of the PCR products were measured at 60°C for 1 min, resulting in the clear identification of all genotypes of SOD2 or GPx1 on a two-dimensional graph.

Statistical analysis

All statistical analyses were performed using SPSS v18 (SPSS France) and JMP v5 (SAS Institute, Cary, NC) softwares. All data were collected from the patients' charts and entered into a computerized database. Results were expressed as mean ± SD. Comparisons were made using Kruskal-Wallis test, Mann-Whitney test and *t*-test when appropriate. A *p*-value below 0.05 was considered as significant.

Results

Clinical data

We studied 24 patients (age = 57.5 ± 10.7 years; 96% men; eight ICM, 16 DCM). Patients' clinical characteristics and cardiovascular risks factors upon admission are reported in Table I.

Upon admission, patients received drugs including statins (37.5%), ACEI (62.5%) and -blockers (5.3%). Statins were significantly more used in patients with ICM than in patients with DCM (80% vs 19%, $p < 0.005$) (Table I).

All patients had a severe CM with a mean LVEDd of 62.6 ± 8.4 mm and a LVEF evaluated at 25.3 ± 8.5% (Table I). The LV pressure was increased in this population with an E/Ea ratio > 18 without any significant difference between both groups.

At the end of the follow-up, LVEF improved to 39.5 ± 13.7% ($p = 0.012$). Three patients died early (<1 month) and one patient at 6 months. These four patients died due to cardiogenic complications and seven patients had iterative hospitalizations for HF. At the term of the follow-up, HF symptoms decreased in six patients and three patients had a normalization of LVEF. Only three patients were treated with beta-blockers, two were subjected to LV resynchronization, one was submitted to an intracardiac defibrillator with a resynchronization and two patients underwent a cardiac transplantation. In the other patients, haemodynamic control was obtained with medical treatment (ACEI, diuretics and nitrate).

Biochemical data

Biological parameters are summarized in Table I and showed a moderate renal impairment (creatinine 150 ± 40 µmol/L), significantly more important for patients with ICM than with DCM (163 ± 49 vs 116 ± 32 µmol/L, respectively; $p < 0.05$). NT-proBNP levels increased at 8540 ± 1703 ng/L and lactate at 4.2 ± 1.1 mmol/L without any significant difference between the two groups. Cholesterol and triglycerides values were within normal range, with total cholesterol slightly higher in ICM than in DCM but without significant difference (4.45 ± 1.52 vs 4.27 ± 1.34 mmol/L, respectively; NS); LDL-cholesterol levels had similar values without any significant difference between both groups (2.61 ± 1.21 vs 2.64 ± 1.03 mmol/L, respectively) (Table I).

Biochemical evaluation of oxidative stress

Assessment of lipid and protein oxidation. Lipid peroxidation was significantly more important in CS patients than in healthy subjects (Table I). Upon admission, erythrocyte and plasma MDA values were significantly increased, 4.00 ± 1.39 nmol/g Hb ($p < 0.05$) and 2.20 ± 0.40 µmo/L ($p < 0.05$), respectively. OxLDL and CP concentration were also significantly increased in patients when compared to controls ($p < 0.05$ for both parameters).

Activities of erythrocyte antioxidants enzymes. Erythrocyte GPx and SOD activities were within usual values, but catalase activity was significantly decreased in patients when compared to controls ($p < 0.05$).

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Table I. Clinical, cardiac parameters, biological and OS parameters of patients at admission.

	Whole population	ICM	DCM
Patients			
Number (men/female)	24 (23/1)	8 (8/0)	16 (15/1)
Mean age (years ± SD)	57.5 ± 10.7	57.1 ± 10.4	57.7 ± 11
Aetiologies of CM (*n* (%))			
Ischemic	8 (33.3)	8 (100)*	0
Toxic	3 (12.5)	0	3
Idiopathic	12 (45.8)	0	12
Myocarditis	1 (4.2)	0	1
Risk factors (*n* (%))			
Diabetes	11 (45.8)	4 (50)*	7 (43.7)
HBP	7 (29.2)	2 (25)	5 (31.2)
Tobacco	9 (37.5)	3 (37.5)	6 (37.5)
Dyslipidemia	8 (33.3)	5 (62.5)*	3 (18.7)
Treatment (%)			
Statins	37.5	80**	19
ACEI	62.5	70	63
β blockers	5.3	10	6
Echocardiographic parameters			
LVEDd mm (mean ± SD)	62.6 ± 8.4	61.4 ± 7.2	63.3 ± 6.9
LVEF % (mean ± SD):	25.3 ± 8.5	29.75 ± 8.8	23.2 ± 7.5
Lipid parameters			
Cholesterol (mmol/L)	4.3 ± 1.4	4.5 ± 1.5	4.3 ± 1.3
LDL-cholesterol (mmol/L)	2.6 ± 1.1	2.6 ± 1.2	2.6 ± 1.0
Other biological parameters			
NT-pro BNP (ng/mL) ($n < 900$)	8540 ± 1703	10 461 ± 3685	7516 ± 1691
Creatinine (µmol/L) ($n < 110$)	150 ± 40	163 ± 49*	116 ± 32
HbA1c (%) ($n < 6$)	7.6 ± 2.0	8.2 ± 1.9	7.4 ± 2.0
CRP (mg/L) ($n < 5$)	7 ± 0.1	7.3 ± 0.1	6.8 ± 0.1
Haemodynamic support duration (h)	122 ± 105	122 ± 73	122 ± 116
Arrhythmias (%)	51	62*	40
Lipid peroxidation controls			
Erythrocyte MDA (nmol/gHb) 1.2 ± 0.4 (0.7–1.6)	4.0 ± 1.4§§	3.22 ± 0.84§§	4.80 ± 1.10§§
Plasma MDA (µmol/L) 1.16 ± 0.14 (1.3–1.9)	2.20 ± 0.40§	2.23 ± 0.40§	2.19 ± 0.50§
OxLDL (U/L) 17 ± 2 (16–18)	49 ± 12§	45 ± 12§	50 ± 12§
Protein oxidation			
Carbonylated proteins (µmol/g protein) < 0.1	0.187 ± 0.02§§	0.185 ± 0.09§§	0.188 ± 0.01§§
Erythrocyte enzymes activities			
Cu,Zn SOD (U/g Hb) 1100 ± 125 (940–1200)	1385 ± 70	1363 ± 233	1410 ± 305
GPx (U/g Hb) 51 ± 11 (40–75)	45 ± 5.0	51 ± 5.0	60 ± 9
Catalase (U/g Hb) 188 ± 32 (150–240)	125 ± 6§§	125 ± 6§§	133 ± 8§§

ACEI, Angiotensin converting enzyme inhibitor; HBP, high blood pressure; LVEDd, Left ventricular end diastolic diameter; Arrhythmias, non sustained or sustained ventricular tachycardia; CRP, C-Reactive Protein; NT-pro BNP, Amino terminal pro-brain natriuretic peptide; HbA1c, Glycated haemoglobin.

Comparison between values in patients at admission and control values obtained in 100 healthy subjects aged 20–45 years without cardiovascular risk factors (diabetes, smoking, hypertension, dyslipidemia): §$p = 0.01$; §§$p < 0.05$; ICM vs DCM: *$p < 0.05$; **$p < 0.005$.

Genotype analysis. In our population, the distribution of MnSOD genotype was respectively 23.5% Ala/Ala variant, 29.5% Ala/Val variant and 47% Val/Val variant. The proportion of ICM was comparable in these three groups, but toxic aetiologies were only found in patients with the Val/Val variant (Table II). The echocardiographic parameters (LVEDd and LVEF) were comparable between the three groups. However, the severity was significantly more important in patients with Val/Val-MnSOD variant when considering NT-proBNP levels (11310 ± 3875 ng/L in Val/Val vs 6486 ± 1375 ng/L in Ala/Ala and 6004 ± 2228 ng/L in Ala/Val patients; $p = 0.04$) and haemodynamic support duration (Val/Val group: 144.6 ± 76.5 h vs Ala/Val variant: 108.8 ± 52.7 and Ala/Ala variant: 52.5 ± 44.6 h; $p < 0.04$ and $p < 0.05$, respectively) (Table II) with a positive correlation (Spearman $\rho = 0.72$, $p = 0.04$). Finally, Val/Val variant correlated with death (Spearman $\rho = 0.63$, $p < 0.02$), but not with morbidity such as HF events or rehospitalization. Lipid peroxidation and protein oxidation were not influenced by MnSOD variants.

Three GPx variants were reported in this study, i.e. the Pro/Pro variant (64%), the Pro/Leu variant (18%) and the Leu/Leu variant (18%). CM aetiologies were comparable in these three groups, especially regarding ischemic aetiology. Idiopathic CM was more frequent in the group homozygous for the Pro/Pro-GPx1 variant. The severity of CM, analysed with LVEDd and LVEF, NT-proBNP and haemodynamic support

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Table II. Comparison of clinical, biological parameters and oxidative stress status in patients in accordance with MnSOD variants.

MnSOD variants	Ala/Ala	Ala/Val	Val/Val
Patients			
Men (%)	87.55	100	100
Mean age ± SD (years)	56.3 ± 12.7	61.4 ± 8.5	56.7 ± 9.5
Distribution, *n* (%)	6 (23.5)	7 (29.5)	11 (47)
Aetiologies of CM (%)			
Ischemic	37.5	40	45.45
Toxic	0	0	27.3*
Idiopathic	62.5	40	27.25
Myocarditis	0	20	0
Risk factors (%)			
Diabetes	18	20	50
HBP	50	20	18.2
Alcohol	12.5	20	0
Dyslipidemia	37.5	40	27.3
Echocardiographic parameters			
LVEDd mm (mean ± SD)	63.4 ± 8.4	64.2 ± 7.2	63.3 ± 6.9
LVEF % (mean ± SD)	25.4 ± 8.5	25 ± 8.8	24.7 ± 7.5
Lipid variables			
Cholesterol (mmol/L)	4.86 ± 1.39	3.62 ± 1.52	3.80 ± 1.34
LDL-cholesterol (mmol/L)	3.05 ± 1.08	2.12 ± 1.21	2.53 ± 1.03
Other biological parameters			
NT-pro BNP (ng/L) ($n < 900$)	6486 ± 1375	6004 ± 2228	11 310 ± 3875*
Creatinine (µmol/L) ($n < 110$)	125.4 ± 30	149.4 ± 39.7§	143 ± 32*
HbA1c (%) ($n < 6$)	7.9 ± 2.0	8.0 ± 1.9	8.1 ± 2.0
Haemodynamic support duration (h)	52.5 ± 44.6	108.8 ± 52.7§	144.6 ± 76.5*
Death/cardiovascular events	0/1	0/3	4*/3
Lipid peroxidation			
Erythrocyte MDA (nmol/gHb)	2.06 ± 1.39	3.92 ± 0.84	2.77 ± 2.9
Plasma MDA (µmol/L)	2.40 ± 0.40	1.88 ± 0.22	2.26 ± 0.50
OxLDL (U/L)	0.48 ± 0.12	0.46 ± 0.12	0.45 ± 0.12
Protein oxidation			
Carbonylated proteins (µmol/g protein)	0.150 ± 0.05	0.181 ± 0.09	0.200 ± 0.10
Erythrocyte enzyme activities			
Cu,Zn SOD (U/g Hb)	1394 ± 252	1500 ± 333.5	1344 ± 202
GPx (U/g Hb)	53.1 ± 5.0	78.7 ± 5.0	70.0 ± 5.2
Catalase (U/g Hb)	125 ± 6	125 ± 6	133 ± 8

HBP, high blood pressure; LVEDd, Left ventricular end diastolic diameter; NT-pro BNP, Amino terminal pro brain natriuretic peptide; HbA1c, Glycated Haemoglobin. Comparison between values inVal/Val group and the other groups: $*p < 0.05$; and between Ala/Val vs Ala/Ala: $^{\S}p < 0.05$.

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duration, was similar in the three groups (Table III). The erythrocyte GPx activity was preserved in the Pro/Pro, Pro/Leu, Leu/Leu groups, with 59.5, 45.2 and 50.6 U/g Hb, respectively (Table III). GPx variants influenced neither GPx activities ($p = 0.22$) nor major adverse cardiac events such as death, HF or rehospitalization ($p = 0.33$).

Discussion

This study reports for the first time genotype polymorphisms of MnSOD and GPx in patients with CS due to severe CM without acute ischemic disease. In this cohort of 24 patients, our data demonstrate: (a) a production of ROS with elevated lipid peroxidation (erythrocyte and plasma MDA; oxidized LDL) and protein oxidation (carbonylated proteins); (b) a preservation of antioxidant erythrocyte enzyme activities; (c) a correlation of Val/Val-MnSOD variant with the severity of CM, with an increase in NT-proBNP levels as well as an increase in inotrope treatment duration and death, but without any association between the Leu-GPx1 variant and the prognosis of CS; and (d) GPx1 and Mn-SOD variant expression were independent of DCM aetiologies.

SOD isoforms have different localizations and functions. Only mechanisms of EC-SOD, the major scavenger against superoxide anion, had been often studied: beneficial effects of EC-SOD require the heparin-binding domain, which mediates binding of EC-SOD to heparin sulphate proteoglycans on cell surface, and this enzyme is more involved in endothelium dysfunction than in mitochondrial changes [12]. Nevertheless, more recently, impaired binding of the EC-SODR_{213G} gene variant to vascular cells has been shown to result in higher levels of EC-SOD in blood. A gene variant of EC-SOD (ECSODR_{213G}) in the heparin-binding domain

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Table III. Comparison of clinical, biological parameters and oxidative stress status in patients in accordance with GPx variants.

GPx variants	Pro/Pro	Pro/Leu	Leu/Leu
Patients			
Men (%)	100	100	92.3
Mean age (years ± SD)	56.7 ± 9.5	62.7 ± 11.1	56.9 ± 9.8
Distribution, *n* (%)	16 (64)	4 (18)	4 (18)
Aetiologies of CM (%)			
Ischemic	25	33.3	38.5
Toxic	0	33.3	15.3
Idiopathic	75	0	46.2
Myocarditis	0	33.3	0
Risk factors (%)			
Diabetes	38	66	50
HBP	23	0	37.5
Alcohol	23	33	14.3
Dyslipidemia	30.7	33	57
Other biological parameters			
NT-pro BNP (ng/L) ($n < 900$)	5002 ± 1703	8470 ± 3685	6506 ± 3621
Creatinine (μmol/L) ($n < 110$)	119.4 ± 29	129.4 ± 23	132 ± 26
HbA1c (%) ($n < 6$)	8.8 ± 2.0	7.9 ± 1.9	7.6 ± 2.0
Haemodynamic support duration (h)	130 ± 84.6	190 ± 92.7	120 ± 116.5
Death/cardiovascular events	3/5	1/1	0/1
Lipid peroxidation			
Erythrocyte MDA (nmol/gHb)	2.06 ± 1.39	3.92 ± 0.84	2.13 ± 0.30
Plasma MDA (μmol/L)	2.40 ± 0.40	1.83 ± 0.40	2.27 ± 0.30
OxLDL (U/L)	0.41 ± 0.13	0.56 ± 0.09	0.49 ± 0.12
Protein oxidation			
Carbonylated proteins (μmol/g protein)	0.125 ± 0.07	0.230 ± 0.09	0.150 ± 0.09
Erythrocyte enzymes activities			
Cu,Zn SOD (U/g Hb)	1395 ± 276	1111 ± 155	1477 ± 219
GPx (U/g Hb)	59.5 ± 5.05	45.2 ± 5.0	50.6 ± 8.9
Catalase (U/g Hb)	137 ± 19.6	118 ± 13	135 ± 19.8
Non enzymatic antioxidants			
GSH (μmol/gHb)	44.8 ± 5.05	78.7 ± 15.2	46.7 ± 8.4
GSSG (μmol/gHb)	103 ± 6	88 ± 9	76 ± 8

HBP, high blood pressure; LVEDd, Left ventricular end diastolic diameter; NT-pro BNP, Amino terminal pro-brain natriuretic peptide; HbA1c, Glycated Haemoglobin.

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which is common, as compared to most gene variants (perhaps 2–5% in humans), does not reduce the antioxidant activity, but binding to cells is impaired [27–29]. Thus, failure of binding of EC-SOD to vessels leads to increased OS, attenuated nitric oxide-induced relaxation of arteries and is associated with increased risk of ischemic heart disease [14]. Although expression of MnSOD is less than Cu/Zn-SOD and EC-SOD in blood vessels, MnSOD plays a critical and protective role against mitochondrial damage during OS [12]. In deficient MnSOD mice, neonatal lethality dramatically increases [30]. MnSOD is up-regulated by OS and transiently up-regulated by atherosclerosis [31]. Moreover, activity of MnSOD in blood vessels is relatively low; localization of MnSOD in mitochondria plays an important role in the protection against OS and limits mitochondrial damage and development of atherosclerosis [32].

The distribution of MnSOD genotypes in our population showed 47% Val/Val homozygotes, 29.5% Ala/Val heterozygotes and 23.5% Ala/Ala homozygotes. This distribution is different in control subjects, with an increase in the Val/Val homozygotes. Recent studies reported that minor allele frequencies of the Val/Val-MnSOD variant in control subjects varied between 9.1% in studies by Gotoh et al. [33] and 24.7% in the studies by Kimura et al. [34]. In patients with hepatocellular carcinoma, Nahon et al. [35] reported a 25% Val/Val homozygotes, 44% Ala/Val heterozygotes and 31% Ala/Ala homozygotes, without any difference in the distribution in Caucasian controls.

In our study, Val/Val-MnSOD variant was significantly associated with an increased severity evaluated by NT-proBNP levels, inotrope treatment duration and mortality in patients with CS. Although the function of the MnSOD polymorphism is not yet well understood, Sutton et al. [16] showed that the Ala-containing MnSOD is more efficiently carried through the inner mitochondrial membrane due to the α-helix of the mitochondrial targeting sequence. The Val allele, by disrupting the α-helix structure of the V-containing MnSOD, which is important for the translocation of the enzyme to the mitochondrial matrix where it exerts its function, causes the protein to be retained at the level of the mitochondrial inner membrane and has been associated with 30–40% lower activity compared with

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the Ala allele after import into isolated mitochondria *in vitro* [16]. In whole cell experiments, A-containing MnSOD exhibit a four-fold higher activity than V-containing MnSOD, due to the degradation by the proteasome of the V-containing MnSOD slowly imported and to a lower mRNA stability of the Val allele, possibly associated with impaired co-translational import [36]. The low MnSOD activity associated with Val variant may lead to increased susceptibility to OS. The expression of MnSOD is variable and inducible by some non-genetic factors such as cytokines and ethanol. It is worth noting that in our population only two patients had alcohol abuse and have stopped intoxication before the onset of this study. However, it remains possible than treatment interferes with OS and then with erythrocyte enzyme activities. Nevertheless, our population was too small to allow a specific analysis of treatment interaction, although some active molecules like calcium antagonists or thiol-containing agents were excluded.

Increased OS in human end-stage HF may result in a specific up-regulation of catalase gene expression as a compensatory mechanism, whereas SOD and GPx gene expression remain unaffected.

GPx 1 is a soluble selenoprotein that reduces H_2O_2 and organic hyderoperoxides to H_2O and the corresponding alcohols [18]. Five GPx isoenzymes have been identified, but GPx1, that is both a cytosolic and a mitochondrial form of GPx, is the most abundant intracellular isoform. The GPx gene is located on chromosome 3p21.3 [37]. To date, four polymorphisms in the human GPx1 gene are known: –602A/G, +2C/T, Ala/Ala and Pro198Leu [36]. Interestingly, this Pro198Leu variant ascribed to the cSNP in exon 2 of the GPx1 gene had a low level of GPx1 activity and is associated with a 40% decrease in activity for the Leu-GPx1 variant and the combination of polymorphisms (602G, +2T) had a 25% decrease in transcriptional activity [38]. It has been reported that a low activity in GPx1 of red blood cells was associated with an increased risk for the onset of cardiovascular events and was involved in the progress of calcification of coronary arterial walls [39]. In our study, we found that distribution of GPx genotypes was 64% Pro/Pro, 18% Pro/Leu and 18% Leu/Leu, but the erythrocyte enzyme activity was comparable between the three groups of patients. In several studies, the distribution of allele frequency of 198Leu variant varied from 17.9% in Japanese type 2 diabetic patients (comparable with our results) to 41% in subjects from the Finnish/Swedish population [39,40]. Some cardiovascular pathologies are associated with a 198Leu-GPx1 variant, especially a risk of macrovascular disease in diabetic patients, increased intima-media thickness of carotid arteries, increased calcium score in coronary artery [39,41], but not a risk of stroke in a case-control study and in stable or unstable angina [15,41]. These studies support the hypothesis that a change in the function of GPx1 would essentially contribute to vascular OS and progression of atherosclerosis rather than to cardiac metabolism. Erythrocyte GPx1 activity had a normal value in our patients. This result is in accordance with the study of Forsberg et al. [40] that reported that erythrocyte GPx1 activity was not significantly altered in groups of patients showing Pro/Pro, Leu/Leu and Pro/Leu genetic variants of GPx1. However, in current smokers and men [15,41], other non-genetic factors are involved in the lower decrease of GPx activity. Erythrocyte GPx activity can be up-regulated under different conditions of OS. Therefore, if one of the variants has an impaired activity, a compensatory up-regulation cannot be excluded [42].

CS seems to alter the mitochondrial metabolism and then cardiac function but endothelial dysfunction seems to be weakly involved in early complications.

Some limitations of this study have to be underlined: (a) the small number (24) of patients; indeed, the inclusion criteria were severe to get a homogeneous population and we only analysed the role of CS without any acute ischemic implication. Thus, it was difficult to increase the number of patients so far as management of patients is now different as compared to the selection period of our patients; (b) MnSOD activity was not assayed; (c) MDA detects lipid peroxidation, as oxygen radicals are able to attack polyunsaturated fatty acids in membranes and lipoproteins, leading to propagation chains of lipid peroxidation. MDA is only one of many aldehydic compounds produced by lipid peroxidation; fluorometric measurements used in this study were more specific than spectrophotometry [24]. Moreover, we brought out the impact of OS by the determination of other markers, such as protein oxidation, oxidized LDL, glutathione and erythrocyte enzymatic antioxidant activities; and (d) OS was measured on peripheral blood samples. Under these conditions, our results did not show the tissue origin of markers of increased lipid peroxidation; both poorly perfused peripheral muscles and the myocardium could have contributed. It is noteworthy that plasma oxidative stress does not exactly reflect mitochondrial OS and further studies would be necessary to better understand the mechanisms involved.

In conclusion, CS was associated with markers of increased OS, i.e. markers of lipid peroxidation and protein oxidation, without any difference depending on CM aetiologies. 198Pro/Leu-GPx1 genetic polymorphism did not influence erythrocyte glutathione peroxidase activity or prognosis of CS. Only the Val-encoding MnSOD allele was significantly correlated with severity and prognosis of CS. Although MnSOD ensures the detoxification of the superoxide anion in mitochondria, a proper balance in the successive action of MnSOD and GPx1 enzymatic activities is required to ensure hydrogen peroxide detoxification.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on Early Online on 9 November 2010.

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