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Methods Paper

Higher frequency of septic shock in septic patients with the 47C allele (rs4880) of the *SOD2* gene☆

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ABSTRACT

Aim: To analyze the effect of the two different versions of the manganese superoxide dismutase gene (*SOD2*) on sepsis. The *SOD2* gene presents the 47C>T single nucleotide polymorphism (SNP; ID: rs4880) which produces MnSOD with different activities. The −9Val MnSOD (47T allele) is less efficient than the −9Ala version (47C allele). During sepsis there are abundance of ROS, high *SOD2* expression and excess of H_2O_2 synthesis. High concentrations of H_2O_2 could affect the sepsis scenario and/or the sepsis outcome.
Methods: We determined the 47C>T single nucleotide polymorphism (SNP) frequencies in 529 critically ill patients with or without sepsis, facing outcome. To collect information on population frequencies, we obtained a pilot 47C>T genotypic and allelic frequencies in a random group of 139 healthy subjects.
Results: We compared the 47C allele carriers (47CC + 47CT genotypes) with 47TT homozygotes and noticed a significant association between 47C allele carriers and septic shock in septic patients ($P = 0.025$). With an adjusted binary multivariate logistic regression, incorporating 47C>T SNP and the main clinical predictors, we showed high SOFA scores [$P < 0.001$, OR = 9.107 (95% CI = 5.319–15.592)] and 47C allele [$P = 0.011$, OR = 2.125 (95% CI = 1.190–3.794)] were significantly associated with septic shock outcome. With this information we presented a hypothesis suggesting that this negative outcome from sepsis is possibly explained by effects on cellular stress caused by 47C allele.
Conclusion: In our population there was a significant higher frequency of septic shock in septic patients with the 47C allele of the *SOD2* gene. This higher 47C allele frequency in septic patients with negative outcome could be explained by effects of higher activity MnSOD on cellular stress during the sepsis.

1. Introduction

The mitochondria milieu represents an abundant source of reactive oxygen species (ROS), such as the superoxide anion ($O_2^{\bullet -}$) that is generated by incomplete reduction of molecular O_2. In the mitochondria, $O_2^{\bullet -}$ is converted into a less reactive species, such as hydrogen peroxide (H_2O_2) by manganese-dependent superoxide dismutase (MnSOD, EC 1.15.1.1) (Weisiger and Fridovich, 1973). The gene that codifies to manganese superoxide dismutase (*SOD2* gene; *locus* 6q25) has a 47C>T single nucleotide polymorphism

Abbreviations: 47C, allele with Cytosine; 47C>T, substition of Cytosine to Thymine at 47 nucleotide of *SOD2* gene; 47T, allele with Thymine; 47TT, homozygote to Thymine allele of *SOD2* gene; 47CC, homozygote to Cytosine allele of *SOD2* gene; Ala, alanine; Ala-9Val, protein variation from 47C>T SNP of *SOD2* gene; APACHE-II, Acute Physiology And Chronic Health Evaluation II; CAT, catalase; eNOS, endothelial nitric oxide synthase; GPx, glutationa peroxidase; H_2O_2, hydrogen peroxide; HSL, São Lucas Hospital; ICU, Intensive Care Unit; IL, interleukin; LOS, length of stay; LPS, lipopolysaccharide; MnSOD, manganese-dependent superoxide dismutase; mtDNA, mitochondrial DNA; MW, Mann–Whitney U-test; n, number; O_2, molecular oxygen; $O_2^{\bullet -}$, superoxide anion; PCR, Polymerase Chain Reaction; ROS, Greactive oxygen species; SD, standard deviation of the mean; SIRS, systemic inflammatory response syndrome; SNP, single nucleotide polymorphism; SOD, manganese-dependent superoxide dismutase gene; SOFA, Sequential Organ Failure Assessment; SPSS, statistical package; ST, Student's t-test; TNF-α, tumor necrosis factor-alpha; Val, valine; X^2, Pearson Chi-squared test.

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(SNP; ID: rs4880) synthesizing MnSOD with different activities. Because this SNP modifies the N-terminal mitochondrial targeting sequence from alanine (Ala; GCT codon) to valine (Val; GTT codon) at position −9 of MnSOD signal peptide (Ala-9Val protein mutation) (Rosenblum et al., 1996), the presence of alanine (−9Ala; 47C allele) is predicted to lead to higher mitochondrial MnSOD activity than the valine (−9Val; 47T allele) isoform (Hiroi et al., 1999). The −9Ala MnSOD (codified by 47C allele) has an alpha-helical structure which is a common conformation of mitochondrial leader signals, while the −9Val MnSOD (codified by 47T allele) loses the alpha-helical structure by a substitution at this residue (Shimoda-Matsubayashi et al., 1996, 1997). Thereby, the −9Val MnSOD had demonstrated to be less efficiently transported into mitochondria and to be significantly less efficient than the −9Ala MnSOD (Hiroi et al., 1999; Shimoda-Matsubayashi et al., 1996).

During sepsis, in response to lipopolysaccharide (LPS), increased levels of tumor necrosis factor-α (TNF-α) and interleukin (IL)-1, coupled to hypoxia leads to an excess of ROS and, due to the need to convert $O_2^{\bullet -}$, the *SOD2* expression is induced (Wong and Goeddel, 1988) increasing both MnSOD and H_2O_2 (Guidot et al., 1993; Liu et al., 2002; Taylor et al., 1995). Usually, the excess of H_2O_2 is rapidly metabolized to O_2 and H_2O by glutathione peroxidase and catalase, but very high H_2O_2 concentrations will induce mitochondrial DNA strand breaks and mitochondrial dysfunction (Kaneko and Inoue, 1998; McDonald and Pan, 1993). The extension of oxidative cell damage caused by the high concentrations of H_2O_2, however, will depend on MnSOD activity and could affect the sepsis scenario and/or the sepsis outcome.

Concerning that the alterations on MnSOD activity causes an inadequate antioxidant defense and mitochondrial dysfunction (Williams et al., 1998), it would be possible to propose theoretically that an excessive efficient MnSOD activity (the 47C allele trait) would result in higher mitochondrial dysfunction due to excess H_2O_2 production. However, Elsakka et al. (2007) studying a sample of 40 septic patients found a reduced frequency of 47T, but not 47C allele, in patients with sepsis compared to healthy controls ($n = 100$). Their pilot findings concluded that the 47C>T *SOD2* biallelic SNP had a functional effect on sepsis: the authors argued that an inefficient targeting of MnSOD (the 47T allele trait) could result in the mitochondrial dysfunction observed in sepsis by inadequate $O_2^{\bullet -}$ conversion.

Thus, we were interested in demonstrate whether 47C or 47T allele carriers could be prone to higher oxidative stress and therefore develop sepsis or worse outcome from sepsis. To analyze the effect of the two different *SOD2* versions on sepsis outcome, we determined the 47C>T SNP frequencies in a sample of 529 well-characterized critically ill patients.

2. Materials and methods

2.1. Design, subjects, and approval

This single center observational retrospective cohort study was conducted with data from random patients admitted to the Intensive Care Unit (ICU) of the São Lucas Hospital (HSL), Brazil, between March 1st, 2002 and November 31st, 2006. The ICU-HSL is a general non-pediatric Medical-Surgery Intensive Care Unit with 13 beds, with 450 admissions/year. We worked on the archived DNA collection from septic and non-septic patients (controls). Patients were followed until death or hospital discharge. Exclusion criteria were HIV-infection, documented immunodeficiency, immunosuppressive therapy, pregnant, or lactating. The control group was 139 random healthy DNA donors from the Paternity Investigation Unit. All subjects were from southern Brazil which is composed by a singular genetic background: majority of subjects with European ethnicity (Portuguese, Italian, Spanish, and German ancestry) and a small amount of individuals with African traits contributing to their genetic pool (Parra et al., 2003; Salzano and Freire-Maia, 1970). The study was approved by the Research Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul (Tel. +55 51 33203345; protocols #03-01732, and #07-03990), and the informed written consent or assent to participate in was obtained from all subjects or patients' surrogates.

2.2. Phenotyping

Patients admitted to the ICU, were diagnosed for sepsis, severe sepsis and septic shock according to the American College of Chest Physicians/Society of Critical Care Consensus Conference definition (Bone et al., 1992). SIRS (systemic inflammatory response syndrome) was defined by the presence of at least two of the following symptoms: fever or hypothermia (core temperature > 38 °C or < 36 °C); tachycardia (> 90 beats/min); tachypnea or hyperventilation (breaths/min > 20 or $PaCO_2$ < 32 mm Hg); leukocytosis (> 12.000 mm^3) or leucopenia (< 4.000 mm^3). Sepsis was defined as SIRS secondary to infection, severe sepsis were sepsis complicated by organ dysfunction and, septic shock if refractory arterial hypotension to fluid replacement, needing vasopressors.

For illness severity evaluation we used the APACHE-II (Acute Physiology And Chronic Health Evaluation II) score (Knaus et al., 1985) obtained on ICU admission day. Organ dysfunction evaluation was according SOFA (Sequential Organ Failure Assessment) (Vincent et al., 1998) score obtained on ICU admission day (SOFA-1) and daily during the first week from the ICU admission and in days 15 (SOFA-15) and 29 (SOFA-29) for patients that stayed in the ICU. Temporal variation comprised length of stay (LOS) in ICU and hospital stay. Mortality was measured in days until death in total hospital stay: clinical endpoints of the study were discharge from the hospital (considered survivors), or death (considered non-survivors). For those patients with multiple ICU admission during the study period, only data from the first entrance was considered. All clinical data were collected and verified by ICU physicians with control ensure.

2.3. Genotyping

Genomic DNA was extracted from leucocytes by a standard method (Lahiri and Nurnberger, 1991). We used previously described genotyping protocols for the determination of 47C>T *SOD2* SNP (rs4880) (Taufer et al., 2005): Polymerase Chain Reaction (PCR) was performed at a total volume of 25 μL with about 10–100 ng of genomic DNA, 1.6 U Taq DNA Polymerase in Taq Buffer (Life Technologies — Brazil Ltda. INVITROGEN Inv. São Paulo, SP, Brazil), final concentration of each dNTP 0.2 mM, and 2 mM $MgCl_2$, 10% DMSO. The exon 2 segment of the *SOD2* gene was amplified using primers sense 5′-GCC CAG CCT GCG TAG ACG GTC CC-3′, and anti-sense 5′-TGC CTG GAG CCC AGA TAC CCC AAG-3′ (Life Technologies — Brazil Ltda. INVITROGEN Inv. São Paulo, SP, Brazil) where the underlined nucleotide represents the deliberate primer mismatches designed to introduce artificial restriction site (Taufer et al., 2005). The PCR was performed on an PTC-100 thermocycler (MJ Research, Inc. Watertown, MA, USA), as follows: an initial denaturation at 95 °C for 6 min, followed by 35 cycles at 95 °C for 1 min, at 60 °C for 1 min, and at 72 °C for 1 min and 30 s. The final extension step was prolonged to 7 min. The 110 bp PCR amplified product (25 μL) was cleaved in appropriated buffer with 10U of the *Hae*III (GibcoBRL®-Life Technologies™, Rockville, MD, USA) at a total volume of 15 μL at 37 °C for 8 h. At least 15% of the samples were subjected to a second, independent PCR restriction fragment length-polymorphism analysis in order to confirm their genotypes.

Based in HapMap, there is an expected high enough prevalence of the 47C>T SNP in our population: Global 47C = 0.48, 47T = 0.52; European 47C = 0.44, 47T = 0.56; Sub-Saharan African 47C = 0.36, 47T = 0.64 (http://www.hapmap.org/), even so in order to have

information about our population frequencies, we obtained a pilot 47C>T genotypic and allelic frequencies in healthy controls. Those data are: 47CC = 0.29, 47CT = 0.41, 47TT = 0.30 and 47C = 0.50, 47T = 0.50; Chi-squared test Hardy–Weinberg equilibrium $P = 0.034$. We did not use healthy subjects as control group because we assumed that the environmental exposure has a crucial influence, therefore we performed comparison among ICU patients. We used a quality control system to ensure genotyping accuracy: sequencing verification of the DNA amplified fragment, black controls, and repetitions. In order to confirm that the 110 bp PCR amplified product really represented the targeted product, we performed a sequence analysis in MegaBase 1000 capillary DNA sequencer (Amersham Biosciences UK Ltd, Chalfont St Giles, Bucks, UK) using the same designed primers. The sequence obtained was submitted to a nucleotide–nucleotide BLAST online alignment (blast, at http://www.ncbi.nlm.nih.gov/BLAST/) with the databases, and we found consensus with the *Homo sapiens* manganese superoxide dismutase gene, exon 2 DNA sequence (GenBank accession # D83493-region 351, GI:1841351) and the sequence exported from chromatogram file. The alignment view was performed in ClustalX program (version 1.8, as described at ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) in multiple alignment modes, with sequences loaded in FASTA format. The lab technicians were blinded to phenotype, and clinical investigators blinded to genotype.

2.4. Statistical analysis

Statistical analyses were conducted using the SPSS 11.5 statistical package (SPSS, Chicago, USA). Continuous variable results are expressed as mean ± standard deviation (SD) and the categorical variables as frequencies and percents. Non-normally distributed scalar variables were analyzed as non-parametric using the Kruskal–Wallis and Mann–Whitney tests. For categorical data, we used the Pearson Chi-squared test. To test Hardy–Weinberg equilibrium, the Chi-squared test was used. To evaluate the influence of individual genotype on the patient outcome, excluding other risk factors that could influence the outcome, we used multiple forward stepwise multivariate logistic regression analysis (Wald method), incorporating patients with and without 47C allele and the clinical predictors. The subjects were classified according to their cutoff value for positive classification in the ROC curve analysis. The primary outcome measure was sepsis. For septic patients the primary outcome measure was septic shock. For inclusion of variables in the multivariate model of logistic regression, we adopted, a correlation between septic shock or mortality and each independent variable at a significance level (P value) lower than 0.25 (Moraes and Souza, 2005). Hazard function analysis by the Kaplan–Meier (Log-rank statistic) procedure was also applied. All reported P values are two-tailed and considered statistically significant when 0.05 or less.

3. Results

We obtained data from 529 patients admitted to the ICU to a maximum of 224 days. Part of these patients was described by Paskulin et al., 2011. Table 1 describes patient profile ($n = 529$) grouped according to sepsis phenotype: patients with ($n = 356$; 67.3%) and without ($n = 173$; 32.7%) sepsis. Among septic patients, 99.7% (355/356) acquired sepsis before ICU admission. The high incidence of sepsis and septic shock was attributed to the nature ICU. Demographic, clinical, and genetic characteristics were stated: the two groups had significant differences in several parameters. The general genotypic and allelic frequencies in ICU sample ($n = 529$) did not differ from the values expected by the Hardy–Weinberg model ($P = 0.893$). The frequencies from sub-samples obtained from patients with or without sepsis show that there were also no deviation from equilibrium [septic: 47CC = 0.25 (89/356), 47CT = 0.49 (176/356), 47TT = 0.26 (91/356) and 47C = 0.50 (354/712), 47T = 0.50 (358/712); $P = 0.833$; non-septic: 47CC = 0.23 (39/173), 47CT = 0.52 (90/173), 47TT = 0.25 (44/173) and 47C = 0.49 (168/346), 47T = 0.51 (178/346); $P = 0.587$]. Comparing patients with and without sepsis in the whole sample ($n = 529$) we did not find significant association between sepsis and 47C>T *SOD2* genotypes ($P = 0.800$) or alleles ($P = 0.722$).

Table 1
Demographic, clinical and genetic profile of 529 critically ill patients with and without sepsis.

Variables	All	With sepsis	Without sepsis	*P*
Patients [*n* (%)]	529 (100)	356 (67.3)	173 (32.7)	
Male [*n* (%)]	285 (53.6)	191 (53.7)	92 (53.2)	0.919^{X^2}
Age [years; mean (SD)]	54.8 (20.0)	56.0 (19.5)	52.3 (20.8)	0.047^{ST}
Admission cause — Medical [*n* (%)]	443 (83.7)	309 (86.8)	134 (77.5)	0.006^{X^2}
APACHE II [mean (SD)]	19.6 (7.9)	21.0 (7.0)	16.5 (8.2)	0.291^{ST}
SOFA-1 [median (min/max)]	6 (0/18)	8 (0/18)	4 (0/17)	0.000^{MW}
SOFA-2 [median (min/max)]	6 (0/18)	7 (0/18)	4 (0/14)	0.000^{MW}
SOFA-3 [median (min/max)]	6 (0/18)	7 (0/18)	4 (0/13)	0.000^{MW}
SOFA-4 [median (min/max)]	6 (0/19)	6 (0/19)	4 (0/14)	0.000^{MW}
SOFA-5 [median (min/max)]	5 (0/20)	6 (0/20)	4 (0/14)	0.000^{MW}
SOFA-6 [median (min/max)]	5 (0/21)	6 (0/21)	3 (0/14)	0.000^{MW}
SOFA-7 [median (min/max)]	5 (0/24)	6 (0/24)	4 (0/12)	0.000^{MW}
SOFA-15 [median (min/max)]	5 (0/19)	6 (0/19)	3 (0/10)	0.000^{MW}
SOFA-29 [median (min/max)]	4 (0/16)	6 (0/16)	3 (0/08)	0.004^{MW}
ICU LOS [days; median (min/max)]	15 (0/125)	15 (0/118)	11 (1/125)	0.000^{MW}
Hospital LOS [days; median (min/max)]	36 (1/242)	36 (1/165)	35.5 (1/242)	0.502^{MW}
47CC [*n* (%)]	128 (24.2)	89 (25.0)	39 (22.5)	0.800^{X^2}
47CT [*n* (%)]	266 (50.3)	176 (49.4)	90 (52.1)	
47TT [*n* (%)]	135 (25.5)	91 (25.6)	44 (25.4)	
47CC + 47CT [*n* (%)]	394 (74.5)	265 (74.4)	129 (74.6)	0.975^{X^2} (a)
47TT + 47CT [*n* (%)]	401 (75.8)	267 (75.0)	134 (77.5)	0.536^{X^2} (b)
47C allele [*n* (%)]	522 (49.0)	354 (50.0)	168 (48.0)	0.722^{X^2}
47T allele [*n* (%)]	536 (51.0)	358 (50.0)	178 (52.0)	
Mortality [*n* (%)]	243 (46.2)	198 (55.9)	45 (26.2)	0.000^{X^2}

47C carriers: 47CC homozygotes and 47CT heterozygotes to 47C>T SOD2 SNP; 47TT patients: 47TT homozygotes; APACHE-II: Acute Physiology and Chronic Health Evaluation II; SOFA: Sequential Organ Failure Assessment; ICU: Intensive Care Unit; Hospital: ICU plus hospital; LOS: length of stay; *n*: number; SD: standard deviation of the mean; ST: Student's *t*-test; MW: Mann–Whitney U-test; X^2: Pearson Chi-squared test; *P* value describes a comparison between septic and non-septic patients; (a) 47CC + 47CT genotypic group versus 47TT homozygotes; (b) 47TT + 47CT genotypic group versus 47CC homozygotes.

Table 2
Adverse outcomes from sepsis in the septic patient's subgroup by 47C>T SOD2 SNP: (A) septic shock; (B) mortality.

A — Septic shock from sepsis	With septic shock	Without septic shock	*P*
Patients [*n* (%)]	252 (70.8)	104 (29.2)	
Male [*n* (%)]	135 (53.6)	56 (53.8)	0.962^{X^2}
Age [years; mean (SD)]	56.1 (18.7)	55.7 (21.5)	0.865^{ST}
Nosocomial infection [*n* (%)]	127 (50.4)	49 (47.1)	0.573^{X^2}
Admission cause — Medical [*n* (%)]	221 (87.7)	88 (84.6)	0.434^{X^2}
APACHE II [mean (SD)]	21.6 (7.0)	19.2 (7.7)	0.004^{ST}
SOFA-1 [median (min/max)]	8 (1/18)	4 (0/13)	0.000^{MW}
SOFA-2 [median (min/max)]	8 (0/18)	4.5(0/15)	0.000^{MW}
SOFA-3 [median (min/max)]	8 (0/18)	5 (0/15)	0.000^{MW}
SOFA-4 [median (min/max)]	7 (0/19)	5 (0/14)	0.000^{MW}
SOFA-5 [median (min/max)]	7 (0/20)	4 (0/18)	0.000^{MW}
SOFA-6 [median (min/max)]	7 (0/21)	4 (0/17)	0.000^{MW}
SOFA-7 [median (min/max)]	7 (0/24)	4 (0/18)	0.000^{MW}
SOFA-15 [median (min/max)]	6 (0/19)	4 (0/16)	0.019^{MW}
SOFA-29 [median (min/max)]	6 (0/16)	4 (0/09)	0.096^{MW}
ICU LOS [days; median (min/max)]	15 (0/118)	13 (2/107)	0.014^{MW}
Hospital LOS [days; median (min/max)]	36 (3/165)	36 (1/156)	0.962^{MW}
47CC [*n* (%)]	65 (25.8)	24 (23.1)	
47CT [*n* (%)]	131 (52.0)	45 (43.2)	0.078^{X^2}
47TT [*n* (%)]	56 (22.2)	35 (33.7)	
47CC + 47CT [*n* (%)]	196 (77.8)	69 (66.3)	0.025^{X^2} (a)
47TT + 47CT [*n* (%)]	187 (74.2)	80 (76.9)	0.536^{X^2} (b)
47C allele [*n* (%)]	261 (51.8)	93 (44.7)	0.086^{X^2}
47T allele [*n* (%)]	243 (48.2)	115 (55.3)	
Mortality [*n* (%)]	161 (64.1)	37 (35.9)	0.000^{X^2}

B — Mortality from sepsis	Non Survivor	Survivor	*P*
Patients [*n* (%)]	198 (55.9)	156 (44.1)	
Male [*n* (%)]	104 (52.5)	87 (55.8)	0.543^{X^2}
Age [years; mean (SD)]	61.5 (16.9)	49.4 (20.4)	0.000^{ST}
Nosocomial infection [*n* (%)]	111 (56.1)	63 (40.4)	0.003^{X^2}
Admission cause — Medical [*n* (%)]	175 (88.4)	133 (85.3)	0.385^{X^2}
APACHE II [mean (SD)]	23.0 (6.8)	18.3 (7.1)	0.000^{ST}
SOFA-1 [median (min/max)]	8 (1/16)	7 (0/18)	0.000^{MW}
SOFA-2 [median (min/max)]	8 (0/18)	6 (0/16)	0.000^{MW}
SOFA-3 [median (min/max)]	8 (0/18)	6 (0/18)	0.000^{MW}
SOFA-4 [median (min/max)]	7 (0/19)	5 (0/17)	0.000^{MW}
SOFA-5 [median (min/max)]	7.5(0/20)	5 (0/18)	0.000^{MW}
SOFA-6 [median (min/max)]	7 (0/21)	5 (0/16)	0.000^{MW}
SOFA-7 [median (min/max)]	7 (0/24)	4 (0/15)	0.000^{MW}
SOFA-15 [median (min/max)]	7 (0/19)	3 (0/16)	0.000^{MW}
SOFA-29 [median (min/max)]	7 (2/16)	3 (0/11)	0.007^{MW}
ICU LOS [days; median (min/max)]	16 (0/107)	14 (2/82)	0.122^{MW}
Hospital LOS [days; median (min/max)]	32 (3/156)	40 (1/165)	0.003^{MW}
Septic shock [*n* (%)]	161 (81.3)	90 (57.7)	0.000^{X^2}
47CC [*n* (%)]	45 (22.7)	43 (27.6)	0.022^{X^2}
47CT [*n* (%)]	111 (56.1)	65 (41.7)	
47TT [*n* (%)]	42 (21.2)	48 (30.8)	
47CC + 47CT [*n* (%)]	156 (78.8)	108 (69.2)	0.040^{X^2} (a)
47TT + 47CT [*n* (%)]	153 (77.3)	113 (72.4)	0.296^{X^2} (b)
With 47C allele [*n* (%)]	201 (50.8)	151 (48.4)	0.533^{X^2}
With 47T allele [*n* (%)]	195 (49.2)	161 (51.6)	

47C carriers: 47CC homozygotes and 47CT heterozygotes to 47C>T SOD2 SNP; 47TT patients: 47TT homozygotes; APACHE-II: Acute Physiology and Chronic Health Evaluation II; SOFA: Sequential Organ Failure Assessment; ICU: Intensive Care Unit; Hospital: ICU plus hospital; LOS: length of stay; *n*: number; SD: standard deviation of the mean; ST: Student's *t*-test; MW: Mann–Whitney U-test; X^2: Pearson Chi-squared test; *P* value describes a comparison between septic and non-septic patients; (a) 47CC + 47CT genotypic group versus 47TT homozygotes; (b) 47TT + 47CT genotypic group versus 47CC homozygotes.

We investigated the genotype frequencies in adverse outcomes (septic shock and mortality) from sepsis in the septic patient's subgroup (Table 2). Demographic, clinical, and genetic characteristics showed significant differences in some parameters. When compared the three genotype groups (47CC, 47CT, 47TT) separately, we found a trend to septic shock ($P=0.078$) and an unadjusted statistic association with mortality ($P=0.022$). When we compared the 47C allele carriers group (47CC + 47CT genotypes) with 47TT homozygotes, a significant positive, unadjusted association with septic shock (74.0 *vs* 61.5; $P=0.025$; OR = 1.78, 95% CI = 1.04–3.03) and mortality was observed (59.1 *vs* 46.7; $P=0.040$; OR = 0.61, 95% CI = 0.36–1.01). In the allele analysis septic shock ($P=0.086$), but not mortality ($P=0.533$), showed a trend towards association with 47C allele.

In order to test whether it would be acceptable or it would just be likely causality of this genetic study, we performed binary multivariate logistic regression to an adjusted analysis, incorporating both 47C carriers and 47TT homozygotes and the main clinical predictors such as age and organ dysfunction (SOFA) to exclude other risk factors that could influence the outcome (Table 3). Taking all patients together ($n=529$), step 2 (final) of the forward stepwise (Wald) method showed that only SOFA [$P<0.001$, OR = 10.677 (95% CI = 6.942–16.422)], and 47C allele [$P=0.016$, OR = 1.748 (95% CI = 1.108–2.758)] were significantly associated with septic shock outcome. Among septic patients ($n=356$), also step 2 of the forward stepwise method showed that only SOFA-day1 [$P<0.001$, OR = 9.107 (95% CI = 5.319–15.592)], and 47C allele [$P=0.011$, OR = 2.125 (95% CI = 1.190–3.794)] were significantly associated with septic shock outcome. In the binary multivariate logistic regression analysis for mortality, among all patients or septic patients we did not find significant association with the 47C>T genotype groups ($P=0.413$ and $P=0.132$, respectively).

To reanalyze the mortality, we also performed a hazard function analysis by Kaplan–Meier analysis using the 47C>T genotype groups as a discriminating factor. Among all patients, we observed that those carrying the 47C allele did not have worse outcome (Log-rank statistic, $P=0.9147$) when compared to the 47TT homozygotes. The same analysis was conducted on patients with only sepsis ($n=356$) and septic shock ($n=252$), and mortality distribution patterns were different although not statistically significant (Log-rank statistic, $P=0.1944$ and $P=0.3250$, respectively).

4. Discussion

Our results in a ICU population indicated the 47C *SOD2* allele carriers (47CT and 47CC genotypes), in comparison with 47TT homozygote group, did not showed association with sepsis. Among septic patients, a significant association between the 47C *SOD2* allele with of septic shock was identified. This last association was supported by a modest predisposing effect of the allele on mortality in septic patients. Based on the functional evidence that 47C allele (−9Ala MnSOD) leads to higher mitochondrial MnSOD activity (Hiroi et al., 1999), our analysis are in accordance with a substantial influence of the 47C allele on the clinical outcome. However, our hypothesis requires validation in additional large cohorts.

Hiroi et al. (1999) examined the mitochondrial processing efficiency of both −9Val and −9Ala MnSOD leader signals and demonstrated that the −9Val version was significantly less efficiently processed than the −9Ala MnSOD. Before these study, some populational studies have suggested an important role of the 47C>T *SOD2* SNP in human diseases. Since the mitochondria are protected from $O_2^{\bullet -}$ by MnSOD enzyme, cells could become susceptible to $O_2^{\bullet -}$-related damages when the activity of MnSOD in the mitochondria is reduced. For instance, individual variability of these enzymes (polymorphisms) leading to lower antioxidant activity in brain cells could be hypothesized to play a role in schizophrenia, as proposed by Akyol et al. (2005). However, still remains some inconsistency. Disease associations in one population cannot be confirmed in others, for example, discrepancies to cancer predisposition in three different North American populations (Egan et al., 2003; Millikan et al., 2004; Wang et al., 2001). In line, the phenotypical influence of 47C>T SNP on sepsis or mortality is also unknown. In our study we did not find any association between sepsis and 47C>T *SOD2* genotypes. Despite our results, a positive association between 47C allele carriers and sepsis was found in a pilot study that compared 40 septic patients (with four 47TT

Table 3
Septic shock outcome risk analysis by binary logistic regression of the forward stepwise (Wald) method: (A) all critically ill patients ($n=529$); (B) septic patients subgroup ($n=356$).

(A) Step	Variable in the equation	*P*	Wald X^2	β	S.E. (β)	Odds ratio* (95% CI)	Percent of correct prediction
1	Category SOFA	<0.001	114.86	2.32	0.216	10.171(6.655–15.545)	74.2
2	Category SOFA	<0.001	116.23	2.37	0.220	10.677(6.942–16.422)	74.2
	47C allele	0.016	5.76	0.56	0.233	1.748 (1.108–2.758)	

Step	Variable not in the equation	*P*	Score
1	Category age	0.589	0.292
	47C allele	0.016	5.809
2	Category age	0.752	0.100

(B) Step	Variable in the equation	*P*	Wald X^2	β	S.E. (β)	Odds ratio* (95% CI)	Percent of correct prediction
1	Category SOFA	<0.001	64.16	2.14	0.267	8.516 (5.042–14.383)	77.7
2	Category SOFA	<0.001	64.82	2.21	0.274	9.107 (5.319–15.592)	77.7
	47C allele	0.011	6.50	0.75	0.296	2.125 (1.190–3.794)	

Step	Variable not in the equation	*P*	Score
1	Category age	0.865	0.029
2	Category age	0.627	0.236
	47C allele	0.010	6.643

47C carriers: 47CC homozygotes and 47CT heterozygotes to 47C>T SOD2 SNP; β: coefficient of regression; S.E.: standard error; 95% CI: 95% confidence interval.
* Odds ratio: classification of a successful septic shock outcome.

homozygotes) with 100 healthy controls (Elsakka et al., 2007). This disagreement between Elsakka's and our results could be related to: 1 — different sample sizes (septic = 356/non-septic = 173 *vs* septic = 40/non-septic = 100); 2 — diverse control groups (ICU patients without sepsis *vs* healthy non-ICU volunteers); and 3 — diverse genetic background of the casuistic (United Kingdom *vs* southern Brazil). Regarding the ethnic origin, subjects from the southern Brazilian population comprise a singular genetic background with the majority of subjects with European origin (Portuguese, Italian, Spanish, and German ancestry) and a smaller amount of individuals with African traits contributing to their genetic pool (Parra et al., 2003; Salzano and Freire-Maia, 1970). In our previous 47C>T analysis with healthy subjects we obtained balanced genotypic frequencies and the allelic frequencies (47C = 0.50, 47T = 0.50) were comparable to other two studies with similar population: 47C = 0.42; 47T = 0.58 (Taufer et al., 2005) and 47C = 0.49; 47T = 0.51 (Gottlieb et al., 2005). Each one were closed to global prevalence of this SNP (source from HapMap: 47C = 0.48; 47T = 0.52). We noticed that our non-septic ICU patients group had a similar allelic frequencies (47C = 0.48; 47T = 0.52) as the healthy subjects.

It was also detected in peripheral blood mononuclear cells from ten healthy subjects that, despite predictions from structural enzyme studies, there was no difference between genotypes in MnSOD activity after LPS exposition (Elsakka et al., 2007). Based on our results we can infer that MnSOD activity might have more influence on worsening an established sepsis than in predisposing it, e.g., the MnSOD variants could affect the susceptibility to septic shock more than to sepsis from critical illness. The literature shows that during sepsis the $O_2^{\bullet -}$ synthesis is increased; uncoupling of electron transport may occur and $O_2^{\bullet -}$ production most likely increases even when $O_2^{\bullet -}$ tension is normal or low (Guidot et al., 1993). Likewise, the MnSOD expression is prone to convert the $O_2^{\bullet -}$ in H_2O_2. For instance, Suliman et al. demonstrated that LPS of Gram-negative bacteria depleted glutathione (GSH) and increased mitochondrial lipid peroxidation in conjunction with increased MnSOD gene expression (Suliman et al., 2003). These factors stimulate cytokine and ROS production causing damage in mtDNA by oxidizing and decreasing copy number.

Even though the present study is associative and does not allow drawing definitive conclusion about cellular mechanisms of oxidation, we could speculate that a more efficient MnSOD activity (the 47C allele trait) results in mitochondrial dysfunction due to higher H_2O_2 production, especially during sepsis, when there is an excess in H_2O_2 combined with higher *SOD2* expression and GSH depletion. This is a preliminary hypothesis, since the complete cellular mechanism cannot be supported by our current data. It is also important to emphasize that no independent association was detected between 47CC homozygotes and adverse outcome from sepsis, possibly caused by the high rates of organ dysfunction in critically ill patients.

We did not measure MnSOD levels or activity to correlate with *SOD2* alleles because that was already done by other studies (Elsakka et al., 2007; Sutton et al., 2005). We recognize that neither haplotyped-based nor cluster-based *SOD2* gene approach can be a limitation and that multiple-marker studies are more precise than the analysis of a single target. Despite this limitation, we were able to detect a significant effect of 47C SOD allele on septic shock, showing that this unique SNP study may be biological reasonable, i.e., there is a plausible effect of gene product on outcome from an inflammatory condition. To answer whether it would be acceptable or just be likely a causality of genetic association study, we performed a binary multivariate logistic regression to an adjusted analysis incorporating the main clinical predictors to exclude other risk factors that could influence the outcome. This last analysis confirmed that a factual association likely exists between genotype and phenotype.

Finally, we propose that further SNP-array investigations should include the 47C>T *SOD2* SNP alone or in combination with other functionally relevant SNP. Broader advanced studies including additional candidate *SOD2* SNPs and genes such as *SOD3*, *SOD1*, *eNOS* (endothelial nitric oxide synthase), *GPx-1* (glutationa peroxidase 1), *GPx-3*, *GPx-4*, or *CAT* (catalase) could also help to refine the understanding about septic shock predisposition.

5. Conclusions

In conclusion, we demonstrated that the 47C>T (Ala-9Val) *SOD2* SNP did not influence sepsis susceptibility, but it was associated with adverse outcome from sepsis: there was a significant higher frequency of septic shock in 47C allele carriers group than in 47TT homozygotes. Our results and our hypothesis suggest that the higher 47C allele carrier frequency in septic patients with negative outcome

is possibly explained by effects of higher activity MnSOD on cellular stress during the sepsis.

Conflict of interest

None.

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