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Effect of 593C > T *GPx1* SNP alone and in synergy with 47C > T *SOD2* SNP on the outcome of critically ill patients

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ABSTRACT

During critical illness and sepsis there is severe antioxidant depletion, and this scenario raises the critical ill patient's mortality risk. Glutathione peroxidase (*GPx*) is one of the first endogenous antioxidant defense enzymes, and it works cooperatively with superoxide dismutase (SOD) and catalase (CAT) to detoxify free radicals from the cellular environment. Genetic studies are important to understand the complexity of human oxidative stress and how the organism responds to an extreme situation such as critically care conditions. Previous studies with a *GPx1* single nucleotide polymorphism (593C > T SNP; rs1050450; protein variant in *GPx1*: Pro198Leu) showed 593T carriers and 593TT homozygotes present higher risk to develop different diseases. We assessed the relationship of the genotype distribution of *GPx1* SNP in critically ill patients with their conditions (organ dysfunction, sepsis, and septic shock) and their outcome. We monitored 626 critically ill patients daily from the ICU (intensive care unit) admission to their discharge from hospital, or death. Our study revealed a significant association between 593TT *GPx1* genotype and mortality; the mortality rate was higher in homozygous 593TT *GPx1* ($N = 94$) when compared with the group of subjects with genotypes 593CT or 593CC *GPx1* ($N = 532$) (52% vs. 38%, $P = 0.009$; $OR = 1.79$; 95% $CI = 1.13–2.85$). Evaluating the subgroup of 293 ICU patients with sepsis, a pooled analysis including two genetic variants *GPx1* and *SOD2* (47C > T SNP, rs4880; protein variant in MnSOD: Ala–9Val) showed a significant difference in relation to progression to septic shock. The frequency of septic shock among septic patients with 593T *GPx1* and 47C *SOD2* alleles ($N = 122$) was higher when compared with septic patients carrying other settings of genotypes ($N = 174$) (78% vs. 66%; $P = 0.028$; $OR = 1.81$; 95% $CI = 1.03–3.18$). Accepting the previously reported functional effects of these two SNPs on *GPx1* and *SOD2* gene expressions and, consequently, on *GPx1* and MnSOD enzyme activities, we believe our results may be considered as an important contribution for the understanding of oxidative imbalance during the critical ill.

1. Introduction

Patients admitted to the Intensive Care Unit (ICU) are described as presenting with a critical and complex pathological condition resulting from severe physiological weakness [1]. Infections and inflammatory processes prolong the length of stay of these patients in the unit and increase their organ dysfunction and mortality rates [2]. Critical illness is characterized by oxidative stress, which is a major promoter of systemic inflammation and organ failure due to excessive free radical production, depletion of antioxidant defenses, or both [3]. In oxidative stress, the level of toxic reactive oxygen intermediates overcome the endogenous antioxidant defenses of the host and damage biologically relevant molecules, such as DNA, RNA, proteins, carbohydrates, and unsaturated fatty acids of the cell membranes [4–7]. Oxidative stress may not be considered an epiphenomenon in the critically ill patient but part of the underlying pathophysiologic events leading to mitochondrial dysfunction and systemic inflammatory response syndrome

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(SIRS), sepsis, and septic shock, which can lead to multiple organ dysfunction syndrome (MODS) [8] and death. During critical illness, the most severe cases of infection (SIRS, sepsis, or septic shock) are associated with the most severe antioxidant depletion [9–11], and this scenario raises the ICU patient's mortality risk. However, even without organ dysfunctions or severe infection (sepsis or septic shock) some ICU patients can have a positive outcome. It can be explained, at least in part, by the individual's genetic constitution, since some genes have polymorphic variants that help the body recover from this critical condition. Otherwise, some genetic variants may predispose the patient to a worse outcome.

Paludo et al. (2013) showed a significant higher frequency of septic shock in septic ICU patients with the 47C allele of the manganese superoxide dismutase gene (*SOD2,* locus 6q25) [12]. The single nucleotide polymorphism (SNP) on *SOD2* gene (47C > T; rs4880; Ala–9Val signal peptide mutation on MnSOD) synthesizes a manganese-dependent superoxide dismutase (MnSOD, EC 1.15.1.1) with different activities; the alanine version of MnSOD (codified by the 47C allele) is predicted to lead to higher mitochondrial MnSOD activity than the valine (codified by the 47T allele) isoform [13]. The higher 47C allele frequency in septic patients with negative outcome found by Paludo et al. [12] could be explained by effects of higher MnSOD activity on cellular stress during sepsis. The human antioxidant endogenous defense system consists of a variety of extracellular and intracellular antioxidants that are able to protect cells and tissues from reactive oxygen species (ROS) [7]. The balance of antioxidant enzymes activity such as SOD, catalase, or glutathione peroxidase (*GPx*) is, therefore, crucial. The defense mechanism against damage by ROS, SOD plus *GPx* or catalase eliminates many damaging oxygen species; SOD catalyzes the dismutation of superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2) plus O_2 [14]. However, the damage caused by H_2O_2 can be removed by *GPx1* which uses glutathione (GSH) as the reducing agent, or by catalase; both glutathione peroxidases and catalase convert H_2O_2 to $O_2 + H_2O$ [15]. When intracellular antioxidants such as SOD and *GPx1* are not able to act perfectly in ICU patients during critical condition, cellular/tissue damage may be higher and the overall patient outcome may be worse.

Since 1957, *GPx1* is known as an erythrocyte enzyme that protects hemoglobin from oxidative injury [16]. It is part of the enzymatic antioxidant defense preventing oxidative damage to DNA, proteins and lipids by detoxifying hydrogen and lipid peroxides. *GPx1* is expressed in almost all tissues, and also is abundant in organs such as kidney and liver [17]. A SNP in the *GPx1* gene (593C > T; rs1050450; Pro198Leu protein mutation on the *GPx1* enzyme) has the 593T allele and the 593TT genotype associated with different diseases (Table 1). This SNP alters the conformation of the *GPx1* enzyme, changing its efficiency; the protein codified by 593T allele has lower enzyme activity when compared to *GPx1* codified by 593C allele [18].

In this study, it was genotyped the 593C > T *GPx1* SNP in 626 ICU patients and further analysis were conducted regarding patient's organ dysfunctions, sepsis, septic shock, and mortality rates. It was also considered the previous 47C > T *SOD2* SNP genotyping of these critically ill patients to evaluate their outcome.

2. Materials and methods

2.1. Design, subjects, and approval

In this study, we included a total of 626 critically ill adult patients from the general intensive care unit (ICU) of São Lucas Hospital, Porto Alegre, RS, Brazil, admitted between March 1st, 2002 and November 31st, 2006. Part of these patients has been described by Fallavena et al. [19] and Fraga et al. [20]. Patients were not eligible if they were diagnosed with HIV-infection or a known immunodeficiency, taking immunosuppressive drugs, pregnant or lactating. All subjects were from southern Brazil, which is composed by a singular genetic background: the majority of subjects are of European origin (Portuguese, Italians, Spanish, and Germans ancestry), and there is a small number of individuals with African traits contributing to their genetic pool [21]. We assumed that the environmental exposure has a crucial influence, this being the reason why healthy subjects were not used as a control group. As a result, we performed comparison among ICU patients only. The study has been approved by the Research Ethics Committee of Pontifical Catholic University of Rio Grande do Sul and the informed written consent or assent to participate in the research was obtained from all subjects or patients' surrogates (CAAE 18473713.6.0000.5336; Process # 330.285).

2.2. Phenotyping

Patients admitted to the ICU were diagnosed with sepsis, severe sepsis and septic shock according to the American College of Chest Physicians/Society of Critical Care Consensus Conference definition [22]. 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 b36 °C); tachycardia (>90 beats/min); tachypnea or hyperventilation (breaths/min > 20 or PaCO2b32 mm Hg); leukocytosis (>12.000 mm^3) or leucopenia (b4.000 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,

Table 1
Recent studies with 593C > T *GPx1* polymorphism in different conditions.

Author	Subjects' origin	N	Risk to	Association with 593T *GPx1* allele
Ratnasinghe et al. 2000 [34]	European	315	Lung cancer	S
Hansen et al. 2009 [26]	European	1154	Colorectal cancer	Ns
Jablonska 2009 [43]	European	405	More selenium in plasma	S
Kucukgergin et al. 2011 [32]	Eurasian	293	Prostate cancer	S
Gurbuzier et al. 2012 [27]	Eurasian	314	Tonsillitis; tonsillar hypertrophy	Ns
Zheikova et al. 2012 [31]	Eurasian	381	Bladder cancer	S
Nahon et al. 2012 [35]	European	200	Hepatitis C with cirrhosis	Ns
Ramprasath et al. 2012 [29]	Asian	824	Diabetic cardiovascular disease	S
Zheikova et al. 2012 [31]	European	210	Coronary artery disease	S
Zhao et al. 2012 [28]	Asian	768	Glioma	S
Johnson Phillips et al. 2013 [30]	North American	585	Depression	S

N: Number of cases + controls.
S: Significant.
Ns: Not significant.

needing vasopressors. For illness severity evaluation we used the APACHE-II (Acute Physiology And Chronic Health Evaluation II) score [23] obtained on ICU admission day. Organ dysfunction evaluation was performed according to SOFA (Sequential Organ Failure Assessment) [24] (Vincent et al. 1998) score obtained on ICU admission day (SOFA-1), on a daily basis during the first week of ICU admission (SOFA-2–SOFA-7) and on 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 were 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 [25]. The genotyping protocol for the determination of 593C > T *GPx1* SNP was as follows: Polymerase Chain Reaction (PCR) was performed at a total volume of 25 μL with about 10–100 ng of genomic DNA, 2.5 μL 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 MgCl2. The segment of the *GPx1* gene was amplified using primers sense 5′-TGT GCC CCT ACG CAG GT-3′, and anti-sense 5′-CCA AAT GAC AAT GAC ACA GG-3′ (Life Technologies Ltda, Brazil. INVITROGEN Inv. São Paulo, SP, Brazil). PCR was performed on Veriti thermocycler from Applied Biosystems as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles at 95 °C for 40 s, 59 °C for 1 min, and 72 °C for 1 min and 30 s. The final extension step was prolonged to 10 min. The 338 bp amplification product was quantified (10–50 ng/μl) by Low Mass (Invitogen-Life Technologies, São Paulo, Brazil) in 1% agarose gel. PCR products were directly sequenced using the same primer sequences in the ABI 3730 XL DNA Sequencer according to the manufacturer's manual. Genotyping was performed by interpretation of chromatogram peaks by sequencing FinchTV software version 1.4.0. We also performed a second independent PCR restriction fragment length-polymorphism analysis in order to confirm some genotypes; the 338 bp PCR amplified product was cleaved in appropriated buffer with 100U of Apa I (GibcoBRL®-Life Technologies™, Rockville, MD, USA) at a total volume of 15 μL at 37 °C for 8 h. We used a quality control system to ensure genotyping accuracy: sequencing verification of the DNA amplified fragment, black controls, and duplicates.

2.4. Statistical analysis

Statistical calculations were performed using the statistical package BioEstat 5.3 (http://www.mamiraua.org.br/pt-br/). Unless otherwise stated, continuous variable results are expressed as a mean and standard deviation (SD), and categorical variables as frequencies and percentage. Means were compared using one-way analysis of variance, and non-normally distributed variables were analyzed as non-parametric using Mann–Whitney test. For the categorical data we used Pearson chi-square test, also used to test for Hardy–Weinberg equilibrium. To evaluate the influence of individual genotype on the patient outcome, excluding other risk factors that could influence the mortality, we used multiple regression analysis incorporating 593C > T genotypes and other clinical predictors. All reported *P* values are two-tailed and considered statistically significant when 0.05 or less. To explore 593C > T *GPx1* and 47C > T *SOD2* SNP data together, we assumed an independent segregation since *GPx1* gene is in chromosome 3 (3p21.3) and *SOD2* gene is in chromosome 6 (6q25). Using a sample of subjects with DNA profile to both *SOD2* and *GPx1* SNPs ($N = 446$), we clustered the patients with simultaneously 593T *GPx1* and 47C *SOD2* alleles ($N = 198$; double genotypes 593TT/47CC; 593TT/47CT; 593CT/47CC; 593CT/47CT) versus the patients with other genotypes setting ($N = 248$; double genotypes 593TT/47TT; 593CT/47TT; 593CC/47TT; 593CC/47CT; 593CC/47CC). We tested other clusters but none of them was informative to detect differences related to genetic variable.

3. Results

We obtained data of the first day to a maximum of 224 days from 626 patients admitted to the ICU. We evaluated the demographic, clinical and genetic profile of 626 critically ill patients clustered according sepsis (74%; 463/626) versus no-sepsis (26%; 163/626), septic shock (52%; 327/626) versus no-septic shock (48%; 299/626), and mortality (40%; 250/626) versus no-mortality (60%; 376/626). We observed no differences in sex, age, or length of stay in ICU. SOFA and APACHE-II scores were significant higher among patients with sepsis, septic shock, and mortality ($P < 0.05$; data no shown). The general genotypic and allelic frequencies in ICU sample ($n = 626$) did not differ from the values expected by the Hardy–Weinberg model ($P = 0.654$); the genotypic frequencies were: 593CC = 0.41, 593CT = 0.44, and 593TT = 0.15. We did not find significant association among the three genotypes comparing patients according sepsis versus no-sepsis ($P = 0.514$) or septic shock versus no-septic shock ($P = 0.750$) groups. However, when they were clustered in mortality *versus* no-mortality groups the patients showed significant difference by CC, CT, and TT genotypes ($P = 0.0397$). We tested dominant, recessive, and co-dominant models for 593C and 593T alleles and observed that the 593C allele had a dominant-like effect in mortality rate. Based on the information that the 593C > T SNP alters the conformation of the *GPx1* enzyme, changing its efficiency, and the protein codified by 593C allele has higher enzyme activity when compared to *GPx1* codified by 593T allele [18], we assumed the dominant model to 593C allele to perform our subsequent analysis.

Table 2 describes patient profile grouped according to 593C > T *GPx1* SNP: patients with 593TT genotype (15%; 94/626) and with 593CT + 593CC genotypes (85%; 532/626). Through the analysis of different variables (organ dysfunction, sepsis, and septic shock) among all critically ill patients no significant difference between the two genotype groups were observed, except in relation to the mortality rate. The rate of ICU mortality was significantly higher in the group of individuals with 593TT genotype than in the group of individuals with 593CT or 593CC genotypes (52% vs. 38%, $P = 0.009$; *OR* = 1.79; 95% *CI* = 1.13–2.85) (Table 2). Analyzing the rates of mortality in the hospital (ICU period plus period in the hospital after ICU discharge), the mortality rate remained significantly higher in the group of individuals with 593TT genotype than in the group with individuals with 593CT or 593CC genotypes (61% vs. 49%, $P = 0.037$). When analyzing the different variables only among septic patients ($N = 463$) or only among patients with septic shock ($N = 327$) we observed no significant difference between the two genotype groups. In order to test whether it would be acceptable or it would just be likely causality of this genetic study, we performed the multiple regression test as an adjusted analysis, incorporating genotypes, sex, age, length of stay in ICU, and APACHE-II scores to exclude other risk factors that could influence the outcome. The analysis showed that only APACHE-II ($t = 6.9850$; $P < 0.001$) was significantly associated with mortality, but the 593TT genotype showed a trend towards association ($t = 1.6318$; $P = 0.1052$).

Using a total sample of 446 ICU subjects with DNA profile to both *SOD2* and *GPx1* genes, we conducted an analysis, the same

Table 2
Critically ill patients' clinical and demographic data according to the 593C > T *GPx1* SNP genotype groups.

Variable	ICU patients	593TT	593CT + CC	*P* value	Test
Frequency (N (%))	626 (100.0)	94 (15.01)	532 (84.98)	0.654	HW
Female (N (%))	300 (47.92)	42 (44.68)	258 (48.49)	0.495	CS
Age (mean (SD))	54.76 (20.03)	55.22 (16.90)	54.68 (20.55)	0.923	MW
SOFA-1 (median (min–max))	6.56 (1–18)	6.85 (1–17)	6.52 (1–18)	0.104	MW
SOFA-2 (median (min–max))	6.51 (1–18)	6.59 (1–18)	6.50 (1–17)	0.882	MW
SOFA-3 (median (min–max))	6.31 (1–18)	6.78 (1–18)	6.24 (1–18)	0.428	MW
SOFA-4 (median (min–max))	6.20 (1–22)	6.58 (1–18)	6.14 (1–22)	0.512	MW
SOFA-5 (median (min–max))	6.11 (1–20)	6.36 (1–20)	6.07 (1–19)	0.654	MW
SOFA-6 (median (min–max))	6.07 (1–21)	6.07 (1–21)	6.07 (1–20)	0.748	MW
SOFA-7 (median (min–max))	5.87 (1–24)	6.03 (1–18)	5.84 (1–24)	0.521	MW
SOFA-15 (median (min–max))	5.95 (1–19)	6.85 (2–15)	5.82 (1–19)	0.656	MW
SOFA-29 (median (min–max))	6.07 (1–14)	4.83 (3–10)	6.30 (1–14)	0.641	MW
APACHE-II score (mean (SD))	19.03 (7.67)	19.56 (7.24)	18.94 (7.44)	0.276	MW
Sepsis (N (%))	463 (73.96)	65 (69.14)	398 (74.81)	0.249	CS
Septic shock (N (%))	327 (52.23)	50 (53.19)	277 (52.06)	0.841	CS
ICU LOS (median (min–max))	17.78 (1–125)	18.64 (1–91)	17.63 (1–125)	0.350	MW
ICU + H LOS (median (min–max))	37.29 (1–242)	33.91 (1–135)	37.90 (1–242)	0.743	MW
Mortality in ICU (N (%))	250 (40.32)	49 (52.13)	201 (38.22)	0.009	CS

593TT = Homozygotes.
593CT + CC = Heterozygotes.
593CC = Homozygotes.
APACHE-II = Acute physiology and chronic health evaluation II.
ICU = Intensive care unit.
ICU + H = ICU plus hospital.
LOS = Length of stay.
N = Number.
SD = Standard deviation.
HW = Pearson chi-square test for Hardy–Weinberg equilibrium.
MW = Mann–Whitney U-test.
HW: Hardy–Weinberg.
CS: Chi-Square.

previous, including the two genotyping. We considered the patients with 593T *GPx1* and 47C *SOD2* alleles ($N = 198$) versus the patients with other genotypes setting ($N = 248$). The result showed that it was not possible to detect a statistically significant difference between the two groups of critically ill patients in any of the variables (sex, age, SOFA and APACHE-II scores, sepsis, septic shock, or mortality). To explore all data, we performed more tests with different approaches using diverse double genotype combination sets, but any important result has been found. When studying the subgroup of septic subjects with double genotype DNA profile of both *SOD2* and *GPx1* genes ($N = 296$), we observed no differences in the mortality rates in sex, age, or SOFA and APACHE-II scores; however there was a significant higher frequency of septic shock in septic patients with 593T *GPx1* and 47C *SOD2* alleles ($N = 122$) when compared to septic patients with other settings of genotypes ($N = 174$) (78% vs. 66%; $P = 0.028$; $OR = 1.81$; 95% $CI = 1.03–3.18$). This combined analyses confirmed the association found by Paludo et al. and also demonstrated an important synergistic relationship between these two oxidative stress-related genes on the phenotype. This synergy between *GPX1* and *SOD2* gene is recognized, but here we are demonstrating a synergy between 593C > T *GPX1* and 47C > T *SOD2* SNP acting on septic patients. We believe this last result demonstrates a simultaneous effect of *GPx1* and *SOD2* gene variation inheritance on the outcome from sepsis.

4. Discussion

Genetic studies are important to understand the complexity of human oxidative stress, and how the organism responds to an extreme situation such as critically care conditions. Glutathione peroxidase (*GPx*) is one of the first endogenous antioxidant defense enzymes, and it works cooperatively to detoxify free radicals from the cellular environment. Previous studies with this *GPx1* SNP (593C > T; rs1050450; Pro198Leu) showed that 593T carriers had higher risk to colorectal cancer [26], tonsillitis [27], glioma [28], diabetic and cardiovascular disease [29] and depression [30]. Other studies noticed that patients with 593TT *GPx1* genotype had higher rates of coronary artery disease [31], prostate cancer [32], bladder cancer [33], lung cancer [34], and hepatitis C with cirrhosis [35] when compared to 593CC *GPx1* homozygotes. Our study revealed a new significant association of 593TT *GPx1* genotype in critically ill subjects with adverse outcome (mortality) when compared to 593CT + 593CC *GPx1* subjects. It seems that the 593TT *GPx1* patient group was more susceptible during the critical state, evolving to worse conditions of health and presenting a higher mortality rate. However it is not so simple to explain the real causes of this relation because complex conditions in ICU patients are caused by many physiological, biochemical, and other intrinsic (genetic) factors, and each factor alone has somewhat of an effect. The fragility of a critically ill patient to sepsis/septic shock or to mortality in an ICU is definitively not triggered by a single gene or factor. Instead genes and multiple factors with small discrete effects are accumulated with comorbidities increasing the risk to sepsis/septic shock or to mortality. In this study it was important to observe that APACHE-II score was stronger ($P < 0.001$) than *GPx1* genotype ($P = 0.105$) to predict mortality in a multivariable analysis. But there was no significant difference in APACHE-II scores between 593TT *GPx1* patient group (with highest mortality rate) and 593CT + 593CC *GPx1* patient group (with lowest mortality rate) ($P = 0.276$). To search for candidate genetic markers to understand a complex condition is a hard task since many genes interfere in a net of intrinsic and extrinsic factors. It may be a challenge to detect the single effect of each gene or each gene variation in this net. Even though we were able to detect a striking association of a single variant in the *GPx1* gene since it was strong enough to appear as a statistically significant factor in the ICU patients' outcome. Based on our results, we believe the 593TT *GPx1* genotype could be associated, even if not directly, with a higher propensity of unbalanced cell oxidative control, thus leading us to suggest the 593C > T *GPx1*

SNP, in its genomic context, as a candidate marker to help understand the differential susceptibility to oxidative imbalance of the critically ill patient.

The human's antioxidant endogenous defense system consists of a variety of extracellular and intracellular antioxidants that are able to protect tissues from reactive oxygen species (ROS) and reactive nitrogen species (RNS) induced injury [7]. SOD, catalase and *GPx* enzymes need to be well coordinate expression and activities in the whole scenario. These antioxidants require trace elements, such as copper, manganese, zinc, iron, and selenium are for their full activity [36]. Selenium, for example, is an essential component of the intracellular antioxidant system as a structural component of the active center of glutathione peroxidase enzymes. Selenium enzymes play a major role in protecting cells against peroxidation, especially lipid peroxidation. Furthermore, selenium seems to play a direct role in the regulation of inflammatory processes and its deficiency is directly correlated to the severity of the disease and the incidence of mortality [37]. Low levels of selenium or other endogenous vitamins and trace elements during the critical illness are also observed due to an escape to the interstitial compartment by capillary leakage, hemodilution, previous insufficient intake, and continuous renal replacement therapies [38]. This loss could explain why intracellular antioxidants such as *GPx1* and SOD enzymes were not able to act perfectly in ICU patients, given that the patient is in critical condition and has loss of elements and ions, even if the ICU patient does not present sepsis. Based on our results we were not able to conclude if 593C > T *GPx1* or 47C > T *SOD2* variants confer more or less susceptibility to fix or decrease the levels of elements and ions, we may only infer if they are influencing the patients' outcome.

During critical illness the most severe cases of infection, like sepsis, are associated to the most severe antioxidant depletion [9–11], and this scenario raises the ICU patient's mortality risk. As reported previously, both 593T and 47C variants change the levels and activities of *GPx1* and *SOD2* enzymes, respectively. *GPx1* enzyme codified by 593T *GPx1* allele has lower enzyme activity than the *GPx1* codified by 593C *GPx1* allele [18]; and the alanine version of MnSOD (codified by 47C *SOD2* allele) is predicted to lead to higher mitochondrial MnSOD activity than the valine (codified by 47T *SOD2* allele) isoform [13]. Thus, these genetic variants could lead the patient to a worse outcome, since intracellular antioxidants MnSOD and *GPx1* are not able to act perfectly in ICU patients during critical condition and cellular/tissue damage may be higher.

Given that the ultimate levels of ROS depend on both dismutation of superoxide anion (O_2^-) into hydrogen peroxide (H_2O_2) by MnSOD and detoxification of H_2O_2 by *GPx1*, and that 593C > T *GPx1* and 47C > T *SOD2* SNPs influence the detoxification pathway of ROS, it is plausible hypothesize the combination of these two gene polymorphisms has a deeper effect on disease risk than a single gene polymorphism [33]. Cox et al. [39] exemplify this in a study in that indicates no association between breast cancer and the two gene polymorphisms when examined independently. However, individuals carrying both 593TT *GPx1* and 47CC *SOD2* genotypes had an increased risk of breast cancer when compared to individuals carrying both 593CC *GPx1* and 47TT *SOD2* genotypes. In another study, Ichimura et al. [40] found that the combination of both 593TT *GPx1* and 47CC *SOD2* genotypes conferred a highest risk for bladder cancer with an odds ratio of 2.69 (95% *CI* = 1.25–5.80; *P* = 0.011). Using the same methodology, Sutton et al. [41] showed in a prospective cohort study alcoholic cirrhotic patients with the high activity MnSOD version (47C *SOD2* allele) and the low activity *GPx1* enzyme (593T *GPx1* allele) presented a higher incidence of hepacellular carcinoma in cirrhosis during follow-up. The authors suggested that these genotypic associations could be explained by an increase of mitochondrial superoxide production by the high dismutation rate of 47T *SOD2* variant and slow detoxification of hydrogen peroxide by the low activity of 593T *GPx1* variant [41,42]. All of these results suggest that these genetic variants may have a synergistic effect on the increase of disease risk.

The combined contribution of multiple genes and environmental factors affect the onset, the evolution and the outcome of complex diseases. Each gene, or gene variation, contributes with its particular small and cumulative effect to the final phenotype. In a previous study Paludo et al. [12] detected an important contribution of the 47C allele of 47C > T *SOD2* SNP on septic shock outcome in ICU septic patients. In their study with 356 septic ICU patients, there was a significant higher frequency of septic shock in septic subjects with the 47C allele of the *SOD2* gene. They compared the 47C allele carriers (47CC + 47CT genotypes; *N* = 252) with 47TT homozygotes (*N* = 104) and noticed a significant association between 47C allele carriers and septic shock in septic patients (78% vs. 66%; *P* = 0.025; *OR* = 1.78; 95% *CI* = 1.04–3.03). The authors suggested the higher 47C allele frequency in septic patients with negative outcome could be explained by effects of higher activity of MnSOD on cellular stress during sepsis. In a combined analyses with both *GPx1* and *SOD2* genetic variants, despite of other variables, we identified a significant higher frequency of septic shock in a group of septic patients with both 593T *GPx1* and 47C *SOD2* alleles when compared to septic patients with other settings of genotypes (78% vs. 66%; *P* = 0.028; *OR* = 1.81; 95% *CI* = 1.03–3.18). Combined analyses can be difficult to process because they need a very large sample size for comparisons and statistic power. However, even with the smaller sample size in this last analysis (*N* = 296), this result could be important to consider future advanced approaches. Combined genetic analysis with more than one SNP are especially interesting because it may demonstrate that the synergic effect of two gene variations could be stronger than single *GPx1* or *SOD2* SNP effects. In association studies, the combined detection of independently segregated SNPs may provide a deeper insight about simultaneously interaction of genes, and how it affects the phenotype.

Another important aspect to consider is epigenetic, that is the study of how molecules binging on DNA can produce significantly different phenotypes as a result of differing biochemical changes that alter gene availability for protein production [44,45]. It can explain why exists differences in patient and population in susceptibility to illness, clinical course, and outcomes. Epigenetic mechanisms, as DNA methylation, can result in increased or decreased gene products. When gene expression is altered, the potential for significant phenotypical alterations to pathology, disease progression, and short- and long-term outcomes exists. Epigenetic regulation, in which gene expression is altered and may significantly impact critical illness outcomes, can occur through direct methylation of DNA cytosine bases resulting in downregulation of genes. Alternatively, demethylation might upregulate expression of genes.

Both 593C > T *GPX1* and 47C > T *SOD2* SNPs has cytosine exchange, that may be affected by mutilation. The prior epigenetic changes of somatic cells could thus have a direct effect on an individual's ability to respond to sepsis in the future [46].

Here, we worked with over 600 patients in critical conditions; we performed comparisons among ICU patients, and we did not use healthy subjects as control group to exclude the crucial influence of environmental exposure. Our study was able to demonstrate the effect of 593C > T *GPx1* and 47C > T *SOD2* SNPs on ICU patient outcome with statistical significance. Accepting the previously reported functional effects of these two SNPs on *GPx1* and *SOD2* gene expressions and, consequently, on *GPx1* and MnSOD enzyme activities, we believe our results may be considered as an important contribution for the understanding of oxidative imbalance during the critical ill.

5. Conclusion

The present study provides statistically founded evidence that the 593C > T *GPx1* SNP alone or in synergic effect with 47C > T *SOD2* SNP is associated with the outcome of critically ill patients. The *GPx1* polymorphism and its genomic context can provide insights for understanding the oxidative stress since its genetic variants have been previously reported to result in changes in enzyme levels and activities.

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