UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

INSTITUTO DE CIÊNCIAS BÁSICAS DA SAÚDE

DEPARTAMENTO DE BIOQUÍMICA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS: BIOQUÍMICA

# Efeitos celulares da variante polimórfica Ala-9Val da MnSOD Humana sobre o estresse oxidativo durante o processo infeccioso: estudo *in vitro.*

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Tese submetida ao programa de Pós-Graduação em Ciências Biológicas: Bioquímica, como requisito parcial para obtenção do grau de Doutor em Bioquímica.

Porto Alegre, 2013

“1 O Senhor é a minha luz e a minha salvação; a quem temerei? O Senhor é a fortaleza da minha vida, diante de quem tremerei?

2 Se malfeitores me atacarem para dilacerar-me, são eles, meus adversários inimigos, que tropeçam e caem.

3 Se um exército vier acampar contra mim, meu coração nada teme. Mesmo que a batalha seja deflagrada, conservo a confiança.

4 Uma coisa pedi ao Senhor, e mantenho meu pedido: morar na casa do Senhor todos os dias de minha vida, para contemplar a beleza do Senhor e zelar pelo seu templo.

5 Pois ele me esconde no seu abrigo no dia da desgraça; ele me esconde no segredo da sua tenda e me levanta sobre um rochedo.

6 E agora minha cabeça domina os inimigos que me cercam. Na sua tenda posso oferecer sacrifícios com a ovação e cantar um salmo ao Senhor.

7 Senhor, escuta meu grito de socorro! Tem piedade de mim, responde-me!

8 Penso na tua palavra: “procure a minha face!” procuro a tua face Senhor.

9 Não me ocultes a tua face! Não afastes com cólera o teu servo! Tu que me socorrestes, não me deixes, não me abandones, Deus da minha salvação.

10 Pai e mãe me abandonaram, o Senhor me recolhe.

11 Mostra-me, Senhor, o teu caminho e conduz-me por uma boa senda, apesar daqueles que me espreitam.

12 Não me entregues ao apetite dos meus adversários, pois contra mim se levantaram testemunhas falsas, cuspindo violência.

13 Tenho certeza: verei os benefícios do Senhor na terra dos vivos.

14 Espera o Senhor; Sê forte, tem coragem; espera o Senhor.”

(Salmo – Capítulo 27: *De David.*)

**AGRADECIMENTO**

A Deus, por permitir que eu fracassasse em fracassar, por ter presenteado a minha vida através da minha esposa e filha.

Ao meu orientador e amigo, José Cláudio Fonseca Moreira, por acreditar, confiar, respeitar, incentivar, orientar-me, por permitir o meu convívio com um grupo espetacular que me ajudou a crescer pessoalmente e profissionalmente.

A minha esposa, Kátia B. Keretzky, por mostrar luz nas trevas, pela confiança incondicional, pelo cuidado, pelo carinho, por ajudar-me a conduzir a nossa família, por não permitir que eu fraquejasse, por fazer eu me sentir o maior homem do mundo pelo simples fato de que tu, Kátia a mulher mais fantástica e maravilhosa do mundo, me ama.

A minha filha, Mariana Paludo, por deixar todos os meus dias ensolarados, por mostrar arco-íris na minha vida, por me permitir conviver com uma princesa.

Aos meus sogros, Aldo M. Keretzky e Jussara B. Keretzky, pelo suporte logístico, por ajudar a cuidar da minha família, pelo amor, pela confiança.

Aos colegas do Laboratório 32, pela ajuda e ensinamentos das diversas técnicas usadas nesta tese.

A todas as pessoas que aceitaram participar voluntariarmente doando material biológico para o desenvolvimento deste trabalho, a todos do Departamento de Bioquímica da UFRGS que de uma maneira direta ou indireta ajudaram.

Ao PPG: Bioquímica (UFRGS) e as agências financiadoras CAPES e CNPq, que me proveram bolsas de estudo durante todo o período desta tese.

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# PARTE I

**RESUMO**

A compreensão da fisiologia e dos mecanismos moleculares da sepse tem sido foco de muitos estudos. As infecções severas, como a sepse, são responsáveis por 10% do total de mortes registradas em Unidades de Tratamento Intensivo em todo o mundo. O desfecho da sepse ocorre devido a influência de fatores ambientais e genéticos, cuja expressão de variantes genéticas suportam ou não este desfecho. Muitos mecanismos estão envolvidos na sepse, incluindo a liberação de citocinas e a ativação de neutrófilos, de monócitos e de células endoteliais. Há associação entre superprodução de óxido nítrico, produção excessiva de radicais livres, depleção de antioxidantes, e déficit energético celular. Enzimas antioxidantes endógenas como a Superóxido Dismutase, a Glutationa Peroxidase e a Catalase protegem a célula do dano oxidativo. A enzima superóxido dismutase dependente de manganês é um potente antioxidante intracelular codificada por um gene (SOD2; 6q25-2) que tem sua expressão induzida por mediadores inflamatórios tais como interleucina 1, interleucina 4, interleucina 6, Fator de Necrose Tumoral – α, lipopolisacarídeos. O gene SOD2 apresenta um polimorfismo de mutação de base $C_{47} \rightarrow T$ no exon 2, o qual resulta na substituição do resíduo 16 ($Ala_{16} \rightarrow Val$) pertencente ao peptídeo sinal da proteína. O objetivo deste trabalho foi estudar o efeito diferencial das variantes -9Ala e -9Val da superóxido dismutase dependente de manganês sobre as células mononucleares de sangue periférico humano (*in vitro*) durante um processo infeccioso (induzido por lipopolisacarídeos), investigando sua implicação: (I) na produção de Espécies Reativas; (II) na atividade e imuno-conteúdo da Superóxido Dismutase dependente de Manganês; (III) na atividade e imuno- conteúdo da Catalase; (IV) na atividade e imuno-conteúdo da Glutationa Peroxidase; (V) na produção de nitrotirosina; (VI) na produção de nitrito/nitrato; (VII) na liberação de Fator de Necrose Tumoral - α; (VIII) na produção de Carboximetil-lisina; (IX) dienos conjugados; (X) no imuno-conteúdo da Poli (ADP ribose) Polimerase; (XI) no imuno-conteúdo do Receptor de Produtos Avançados de Glicação; (XII) no imuno-conteúdo da Proteína de Choque Térmico; (XIII) no imuno-conteúdo do Fator Nuclear κB; (XIV) no dano ao DNA celular; (XV) na determinação das defesas antioxidantes totais não enzimáticas. Os resultados demonstraram que o polimorfismo Ala-9Val participa na regulação do ambiente redox celular, e que o alelo 47C permite que as células no estado basal (sem lipopolisacarídeos) respondam com mais eficiência ao estresse oxidativo celular. Este alelo apesar de produzir mais espécies reativas também aumenta o mecanismo de defesa antioxidante. Porém, quando em uma doença que produza estresse oxidativo, no caso a sepse, o alelo 47C torna o ambiente intracelular pró-oxidativo podendo agravar a condição celular. Em suma, os dados aqui apresentados sugerem que o polimorfismo Ala-9Val é um alvo promissor para novos estudos com o objetivo de usar marcadores genéticos para direcionar a terapia necessária para cada paciente.

**Palavras-chave:** Sepse; Lipopolisacarídeos; Polimorfismo Ala-9Val do gene SOD2; Espécies Reativas.

**ABSTRACT**

The understanding of the physiology and of molecular mechanisms of sepsis has been focus of many studies. The severe infections, as the sepsis, are responsible for 10% of total of deaths registered in Intensive Care Units all over the world. The outcome of sepsis happens due to influence of environmental and genetic factors, whose the expression of genetic variants supports or not this outcome. Many mechanisms are involved in sepsis, including the cytokines liberation and the neutrophils activation, of monocytes and of endothelial cells. There is association among overproduction of nitric oxide, excessive production of free radicals, depletion of antioxidants, and cellular energy deficit. Endogenous antioxidant enzymes as Superoxide Dismutase, Glutathione Peroxidase and Catalase protect the cell of oxidative damage. The manganese superoxide dismutase enzyme it is a potent antioxidant intracellular codified by a gene (SOD2; 6q25-2) that has her expression induced by the inflammatory mediators such as interleukin 1, interleukin 4, interleukin 6, tumor necrosis factor – α, lipopolysaccharide. The SOD2 gene presents a single-nucleotide polymorphism $C_{47} \rightarrow T$ in the exon 2, which results in the substitution of the residue 16 (Ala16 Val) belonging to the signal peptide of the protein. The aim of this work was to study the differential effect of the variants -9Ala and -9Val of manganese superoxide dismutase on the Peripheral Blood Mononuclears Cells human (in vitro) during an infectious process (induced by lipopolysaccharide), investigating her implication: (I) in the production of Reactive Species; (II) in the activity and immunocontent of Manganese Superoxide Dismutase; (III) in the activity and immunocontent of Catalase; (IV) in the activity and immunocontent of Glutathione Peroxidase; (V) in the nitrotyrosine production; (VI) in the nitrite/nitrate production; (VII) in the production of tumor necrosis factor - α; (VIII) in the production of carboxymethyl lysine; (IX) conjugated dienos; (X) in the immunocontent of the Poly (ADP-ribose) Polymerase; (XI) in the immunocontent of the Receptor for Advanced Glycation Endproducts; (XII) in the immunocontent of Heat Shock Protein; (XIII) in the immunocontent of the Nuclear Factor kappa B; (XIV) in the damage to cellular DNA; (XV) in the determination of the non-enzymatic antioxidant cellular defenses. The results demonstrated that the polymorphism Ala-9Val it participates in the regulation of the cellular redox environment, and that the 47C allele allows that the cells in the basal state (without lipopolysaccharide) they answer with more efficiency to the stress oxidative cellular. This allele in spite of producing more RS also increases the mechanism of antioxidant defense. However when in a disease that produces oxidative stress, in the case the sepsis, the 47C allele turns intracellular environmental pro-oxidative could worsen the cellular condition. In summary, the data presented here suggest that the polymorphism Ala-9Val is a promising target for new studies with the goal of using genetic markers to guide therapy required for each patient.

**Key-words:** Sepsis; Lipopolysaccharides; *SOD2* Ala-9Val polymorphism; Reactive Species.

**LISTA DE ABREVIATURAS**

AIF - Fator Indutor Apoptose

Ala - Alanina

ATP - Adenosina Trifosfato

$Ca^{2+}$ - Cálcio

CAT – Catalase

CD14 – Receptor de Superficie (*Cluster of Differentiation*)

CML - Carboximetil-lisina

CuZnSOD - Superóxido Dismutase Dependente de Cobre/Zinco

cyt c - Citocromo C

DCFH-DA - 2',7'-diclorofluoresceína-diacetato

DNA - Ácido Deoxirribonucleico

EC-SOD - Superóxido Dismutase Extracelular

eNOS - Óxido Nítrico Sintase endotelial

ER - Espécies Reativas

ERO - Espécies Reativas de Oxigênio

GPx - Glutationa Peroxidase

GSH - Glutationa

HMGB-1 - Proteína de Alta Mobilidade Box 1 (H*igh Mobility Group B-1 protein*)

$H_2O_2$ - Peróxido de Hidrogênio

HSP – Proteína de Choque Térmico (*Heat Shock Protein*)

IFN- γ - interferon γ

IKK - Proteína Kinase do Inibidor κB

IL - Interleucina

IκBα - Inibidor κB

iNOS - Óxido Nítrico Sintase induzida

LPS - lipopolisacarídeos

MAPK – Quinase de Proteínas ativadas por Mitógenos (*Mitogen-activated Protein Kinase*)

MnSOD - Superóxido Dismutase dependente de Manganês

mRNA - Ácido Ribonucléico Mensageiro

mtNOS - Óxido Nítrico Sintase Mitocondrial

NFκB – Fator Nuclear κB (*Nuclear Factor* kappa B p50/p65)

nNOS - Óxido Nítrico Sintase neuronal

NO - Óxido Nítrico

$O_2^{\cdot -}$ - Superóxido

$ONOO^-$ - Peroxinitrito

PAF - Fatores de Agregação Plaquetária

PARP - Poli (ADP ribose) Polimerase

PBMC - Células Mononucleares de Sangue Periférico (*Peripheral Blood Mononuclear Cell*)

PUCRS - Pontifícia Universidade Católica do Rio Grande do Sul

RAGE - Receptor de Produtos Avançados de Glicação (*Receptor for Advanced Glycation Endproducts*)

RL - Radicais Livres

SDMO - Síndrome da Disfunção de Múltiplos Órgãos

SNP – Polimorfismo de Único Nucleotídeo (*Single Nucleotide Polymorphisms*)

SOD - Superóxido Dismutase

SOD2 - gene humano da Superóxido Dismutase dependente de Manganês

TAK1 - Quinase Ativada pelo Fator de Crescimento e Diferenciação 1

TLR – Receptor tipo Toll (*Toll-like receptors*)

TNF-α – Fator de Necrose Tumoral - α (*Tumor Necrosis Factor* α)

TNFR - Receptor de Fator de Necrose Tumoral

UTI - Unidade de Terapia Intensiva

UTIG-HSL-PUCRS - UTI Geral do Hospital São Lucas da PUCRS

Val – Valina

# INTRODUÇÃO

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### *SEPSE*

### *Epidemiologia*

Os pacientes internados em Unidade de Terapia Intensiva (UTI) são caracterizados por apresentarem um quadro patológico crítico e complexo, decorrente de fragilidades fisiológicas graves e responsáveis pela elevada taxa de mortalidade. A sepse é a causa mais comum de admissão em UTI não coronarianas [Henkin, *et* al., 2009; Levy, *et al*., 2010] com uma estimativa de 750 000 casos de sepse severa por ano nos Estados Unidos [Vincent, 2008; Angus, 2010] bem como é a principal causa de morte nas UTI com incidência de 30% em sepse grave, e acima de 50% em choque séptico [Silva, *et al.*, 2004; Sales Júnior, *et al*., 2006; Vincent, 2008; Henkin, *et* al., 2009; Koenig, *et al*., 2010; Levy, *et al*., 2010; Wang, *et al*., 2010]. A respeito dos progressos no diagnóstico e no tratamento das doenças infecciosas, a incidência de sepse grave aumentou 91,3% nos últimos 10 anos associada com elevada taxa de mortalidade, que permanece inalterada no período [Koenig, *et al*., 2010]. O aumento das infecções causadas por bactérias resistentes a antibióticos e o desenvolvimento de tecnologias de manutenção de vida, com o uso de procedimentos e dispositivos invasivos, podem explicar esse fato [Sands, *et al.,* 1997; Alberti, *et al.,* 2002; Turnidge, 2003; Weber, *et al*., 2008]. Esta alta mortalidade incita intensivas pesquisas no desenvolvimento de novas terapias coadjuvantes no acompanhamento do paciente séptico e no conhecimento do quadro patológico. Nesse sentido, durante a última década, diversos trabalhos foram realizados na tentativa de testar a eficácia de agentes que modulam a resposta do hospedeiro para a infecção [Alberti, *et al.,* 2002; Patel, *et al*., 2003; Cariou, *et al*., 2004; Russel, 2008; Vincent, *et al*., 2011; Kak, *et al*., 2012; Mayeux e MacMillan-Crow, 2012; Rocha, *et al*., 2012].

***Conceitos fundamentais para a definição do quadro séptico***

A compreensão da fisiopatologia e dos mecanismos moleculares da sepse tem sido foco de muitos estudos durante a última década. Responsável por 10% do total de mortes registradas em todo o mundo, as infecções severas, como a sepse, são consideradas as principais causas dos óbitos em Unidades de Tratamento Intensivo onde, segundo as estatísticas, o quadro clínico de 40% dos pacientes internados evolui para choque séptico [López-Bojórquez, *et al.,* 2004; Henkin, *et* al., 2009; Hall, *et al*., 2011].

O episódio séptico leva em conta inúmeros fatores. Ele pode atingir indivíduos de qualquer idade ou população, mas sempre tem início por um processo infeccioso causado por bactérias Gram-positivas ou Gram-negativas, fungos (principalmente *Candida* sp.) ou vírus [Tsiotou, *et al.,* 2005].

Definições concisas são necessárias para melhorar a habilidade de um diagnóstico preciso, monitoramento e um posterior tratamento para com os pacientes sépticos. Assim, segundo Tsiotou *et al.* (2005), referiu-se as definições a seguir:

- **Infecção** é definida como processo patológico causado por uma invasão de micro-organismos patogênicos ou potencialmente patogênicos em tecidos estéreis, fluidos ou cavidades corporais;
- **SIRS** (Síndrome Sistêmica da Resposta Inflamatória) é diagnosticada como uma combinação de sinais clínicos, apresentando pelo menos, dois dos critérios a seguir: (I) Febre, temperatura corporal >38ºC ou hipotermia, temperatura corporal <36ºC; (II) Taquicardia, freqüência cardíaca >90 bpm; (III) Taquipnéia, freqüência respiratória >20 irpm ou $PaCO_2$ <32 mmHg; (IV) Leucocitose ou leucopenia, Leucócitos >12.000 cels/$mm^3$ ou <4.000 cels/$mm^3$, ou presença de >10% de neutrófilos de formas jovens (bastões);

- **Sepse** é definida como SIRS induzida por uma infecção;
- **Sepse Grave** é a sepse agravada por disfunção orgânica, hipoperfusão tecidual, ou hipotensão;
- **Choque Séptico** é caracterizado por uma contínua hipotensão arterial com pressão sistólica inferior a 90mmHg, ou uma redução superior a 40mmHg partindo de uma linha basal, na ausência de outras causas para hipotensão.

***Mecanismo de Transdução de Sinais na Sepse***

O sistema imunológico é muito complexo. O entendimento de como ele reconhece trechos de moléculas produzidas pelo organismo (próprio) das não produzidas pelo organismo (não próprio) inibe a resposta auto-imune e ao mesmo tempo permite a reação do hospedeiro contra os invasores [Levy, *et al.,* 2003; López-Bojórquez, *et al.,* 2004].

A sepse inicia por uma resposta inflamatória que direta e indiretamente causa dano celular que se difunde pelo tecido. Bactérias Gram-positivas e Gram-negativas, vírus e fungos têm moléculas chamadas de padrões moleculares associados a patógenos que se ligam a receptores de reconhecimento padrão (TLR) na superfície das células do sistema imune, como exemplo, os lipopolisacarídeos (LPS) de bactérias Gram- negativas que se ligam ao receptor de superficie CD14 e que são reconhecidos pelos TLR-4, e os peptidioglicanos de bactérias Gram-positivas reconhecidos pelos TLR-2. Nestes exemplos, após as ligações com TLR-4 ou TLR-2, há a ativação da proteína quinase do inibidor κB (IKK) que irá fosforilar o inibidor κB (IκBα) associado com o heterodímero Fator Nuclear kappa B (NFκB) p50/p65 (Figura 1). Esta fosforilação libera o heterodímero e sinaliza o IκBα para a degradação através do proteassomo, p50/p65 livres são translocados para o núcleo onde irão iniciar a transcrição de vários genes mediadores da resposta imune e inflamatória como citocinas, moléculas de adesão entre outros [Zingarelli, *et al.*,2005,

Russell, 2006].

Citocinas como fator de necrose tumoral α (TNF-α), interleucina (IL)-1β, IL-6, IL-8, IL-12 e interferon γ (IFN- γ) são fatores solúveis que atingem outros tipos celulares, promovendo alterações internas em tais células além de ativarem linfócitos T que também secretam tais substâncias e participam no combate de células tumorais [Levy, *et al.,* 2003; López-Bojórquez, *et al.,* 2004; Hoesel e Ward*,* 2004].

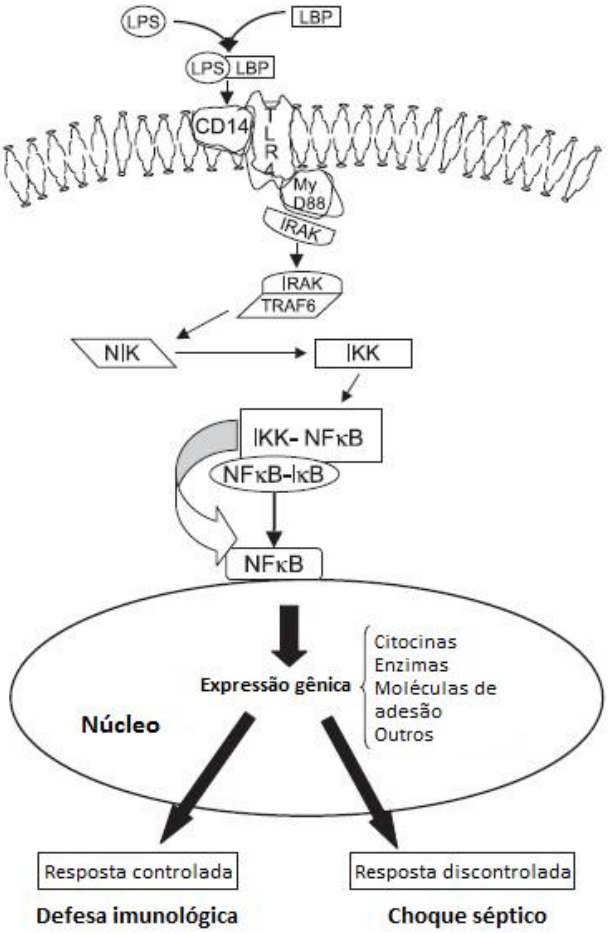


Figura 1. Diagrama esquemático da via de sinalização da resposta inflamatória do hospedeiro induzida por LPS. Adaptadas de Victor, e*t al*., 2004.

***Disfunção Fisiopatológica na Sepse***

Além das citocinas, também ocorre liberação de óxido nítrico (NO), o qual causará hipotensão (relaxamento endotelial) e ativação de fatores de agregação plaquetária (PAF) [Hoesel e Ward, 2004] (Figura 2). A atividade destes compostos pode conduzir à coagulopatia, o que dificulta a perfusão tecidual [Gomez-Jimenez, *et al.,* 1995], causa queda significativa da pressão arterial e causa redução do débito cardíaco. A hipotensão arterial é resultado da combinação de vasodilatação periférica e da síndrome de extravasamento vascular [Landry e Oliver, 2001]. Além da hipovolemia, que contribui para queda do débito cardíaco, existe uma alteração da função do músculo cardíaco, conhecida como depressão miocárdica [Krishnagopalan, *et al.,* 2002].

A vasodilatação periférica acentuada na sepse é clinicamente caracterizada como choque séptico onde se tem um alto trabalho cardíaco e uma baixa resistência vascular sistêmica [Parrillo, *et al*.,1990], porém, o choque não pode ser entendido apenas como hipotensão arterial, e sim por uma inadequada suplementação de oxigênio aos tecidos, secundário a graves distúrbios perfusionais [Task Force of The American College of Critical Care Medicine, 1999; Jindal, *et al*.,2000; Backer, 2006]. O meio pelo qual o oxigênio chega aos tecidos é através da microcirculação. Estudos sugerem que a microcirculação seja o maior sítio de ataques durante a sepse e o choque séptico, modulados pelo endotélio [Hinshaw, *et al*., 1996; Sielenkämper, *et al*., 2002; Kanoore Edul, *et al*., 2011; Backer, *et al*., 2012].

As células endoteliais são responsáveis pela homeostasia do tônus vascular e pelo controle local do fluxo sanguíneo (através da via de coagulação). Elas também influenciam a vazão de fluidos e proteínas do plasma para os tecidos, controlam a ativação de leucócitos, bem como o acúmulo ou o extravasamento desses para os tecidos, por meio da expressão de moléculas de adesão. O endotélio é a principal fonte e alvo da inflamação, tendo um

papel chave na sepse severa e na síndrome da disfunção de múltiplos órgãos (SDMO) [Hack e Zeerleder, 2001; Aird, 2003].

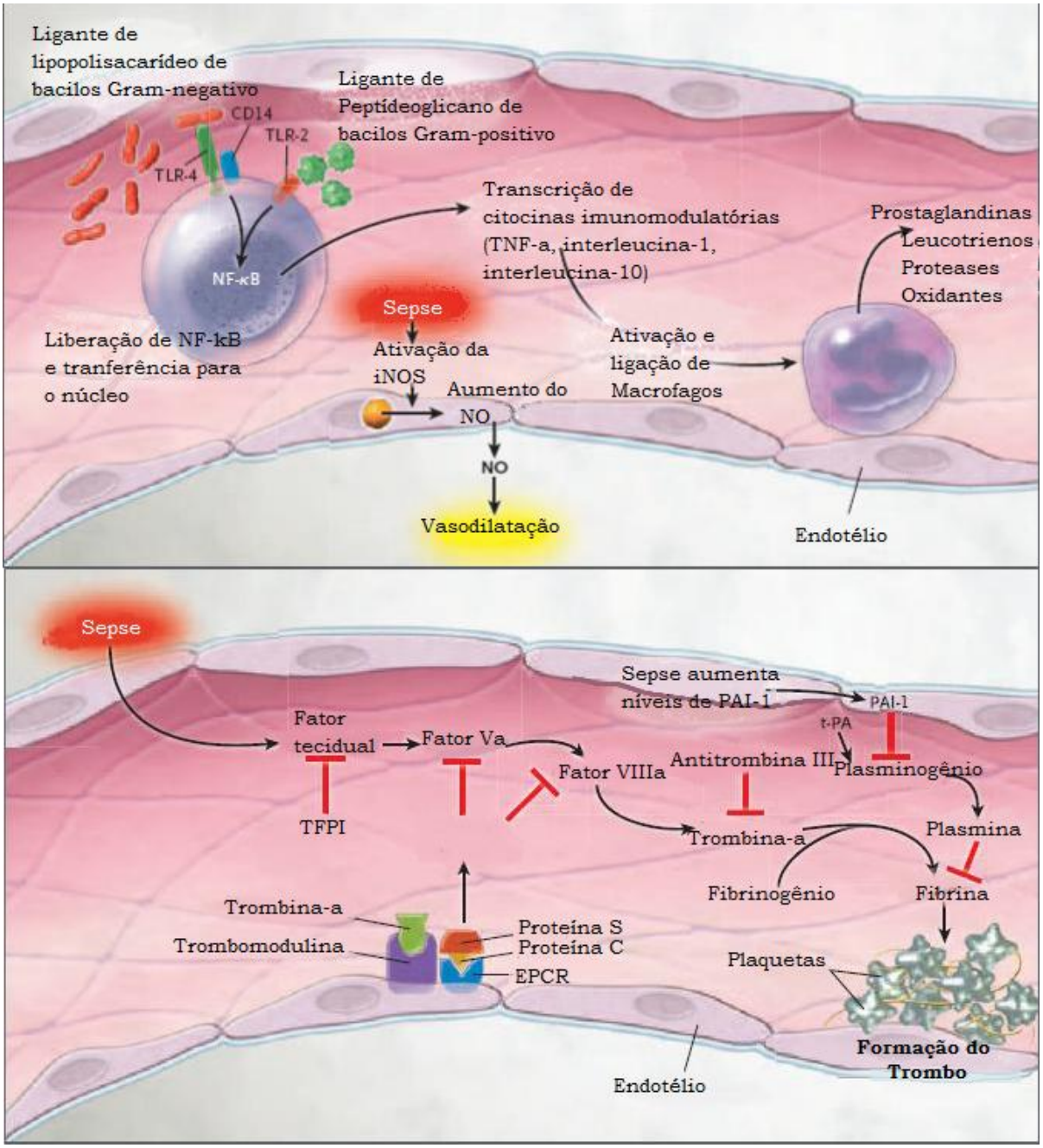


Figura 2. Ilustração da resposta inflamatória rápida iniciada pela sepse que direta ou indiretamente provoca lesão tecidual generalizada, bem como inicia a coagulação através da ativação do endotélio aumentando a expressão do Fator Tecidual. Adaptadas de Russell, 2006.

***Estresse Oxidativo na Sepse***

As espécies reativas de oxigênio (ERO) possuem várias funções no controle fisiológico da célula, sendo contidas e mantidas por um mecanismo redox de homeostasia [Thannickal e Fanburg, 2000; Droge, 2002]. Elas participam da sinalização entre as células de defesa [Forman e Torres, 2001], bem como promovem a interação entre as células endoteliais na microcirculação [Cooper, *et al*., 2002]. Embora a produção de ERO seja essencial para conter a infecção, sua superprodução contribui diretamente para o dano endotelial e dos tecidos através da peroxidação lipídica e do dano ao ácido deoxirribonucleico (DNA) celular [Albuszies e Brückner, 2003].

O maior regulador intracelular do sistema redox é a glutationa (GSH), com a ação via oxidação reversível de um grupo tiol ativo [Arrigo, 1999] e com o NFκB como um de seus reguladores. O NFκB pode ser ativado via TLR ou por elevados níveis de peróxido de hidrogênio ($H_2O_2$) [Schmidt, *et al*., 1995; Khan e Wilson, 1995]. Está bem descrito que as ERO participam do controle da sinalização da transdução de sinal via ativação das quinases de proteínas ativadas por mitógenos (MAPK) [Han, *et al*., 2003], responsáveis por muitas rotas metabólicas, incluindo as que participam da resposta inflamatória. Esta rede de sinalização, ou seja, vários pontos plenamente interligados permitem diferentes tipos de combinações para propiciar diferentes níveis de resposta. O que irá definir o grau de resposta para diferentes sinalizações (desde uma sinalização de hipóxia até uma ativação de apoptose ou necrose) será o desequilíbrio entre a exposição aos oxidantes e a proteção dos antioxidantes [Forman e Torres, 2001].

Durante a sepse há várias possíveis fontes de produção de ERO, tais como: cadeia respiratória mitocondrial [Handy, 2005]; protease mediada pela enzima xantina oxidase [Chatterjee, *et al*., 2011]; granulócitos e outros fagócitos ativados pelo Sistema complementos, bactérias, endotoxinas, enzimas lisossomais, etc. [Huber-Lang, *et al*., 2002;

Victor, e*t al*., 2004]. A disfunção mitocondrial tem um papel central na síndrome da sepse [Callahan e Supinski, 2005; Dare, *et al*., 2009]. Uma desordem no metabolismo energético celular parece ser a causa mais provável para a disfunção celular e, consequentemente, para a disfunção orgânica típica do paciente que apresenta um estado crítico de saúde.

Enzimas antioxidantes endógenas como a Superóxido Dismutase (SOD), a Glutationa Peroxidase (GPx), e a Catalase (CAT) protegem a célula do dano oxidativo [Van Remmen, *et* al., 1999; Klivenyi, *et al*., 2000; Ando, *et al*., 2008].

SOD catalisa a dismutação do superóxido ($O_2^{\cdot -}$) para peróxido de hidrogênio (H2O2):

**Equação 1:** $O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$

A enzima CAT catalisa a degradação do $H_2O_2$. Na reação, uma das moléculas de $H_2O_2$ é oxidada a oxigênio molecular ($O_2$) e a outra é reduzida à água:

**Equação 2:** $2\ H_2O_2 \xrightarrow{CAT} O_2 + H_2O$

A GPx é uma enzima selênio-dependente que catalisa a redução do $H_2O_2$ e hidroperóxidos orgânicos para água e álcool, usando a glutationa como doador de elétrons:

**Equação 3:** $2\ GSH + H2O2 \xrightarrow{GPx} GSSG + H_2O$

**Equação 4:** $2\ GSH + ROOH \xrightarrow{GPx} GSSG + ROH + H_2O$

***Superóxido Dismutase Dependente de Manganês (MnSOD)***

A SOD (EC 1.15.1.1) possui um grande potencial terapêutico para pacientes críticos [Salvemini e Cuzzocrea, 2003]. SOD é uma família de metaloproteínas onipresentes que catalisam a reação de dois ânions de $O_2^{\cdot -}$ com a formação de $H_2O_2$ e $O_2$ [Nordberg e Arnér, 2001]. Três tipos distintos de SODs foram identificados em células humanas [Zelko, *et al*., 2002]: 1) proteína homodimérica citosólica superóxido dismutase dependente de

cobre/zinco (CuZnSOD) [Crapo, *et al*., 1992], 2) proteína homotetramérica localizada na matriz mitocondrial superóxido dismutase dependente de manganês (MnSOD) [Wan, *et al*., 1994], e 3) proteína homotetramérica extracelular superóxido dismutase extracelular (EC-SOD) [Folz e Crapo, 1994] (Figura 3).

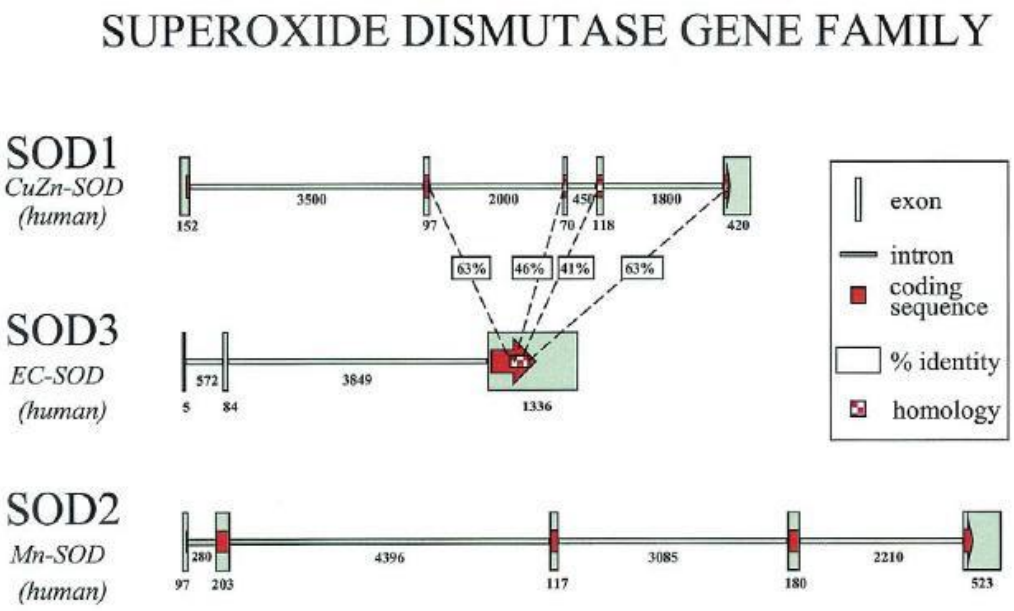


Figura 3. Organização genômica dos três membros conhecidos da família da enzima SOD humana, adaptadas de Zelko, *et al*., 2002.

A MnSOD codificada por um gene nuclear é encontrada na sua forma ativa na matriz mitocondrial [Weisiger e Fridovich, 1973]. A proteína possui 222 aminoácidos [Beck, *et al*., 1987], sendo que os 24 primeiros aminoácidos pertencem a região do peptídeo sinal [Ho e Crapo, 1988] (Figura 4) e, após ser sintetizada no citosol, sofre modificações pós-transcricionais para ser transportada à mitocôndria [Wispe, *et al*., 1989]. Trabalhos com ratos *knockout* para a MnSOD mostraram que esta isoforma é essencial para a vida [Li, *et al*., 1995; Lebovitz, *et al*., 1996], e hipotetizam que uma regulação intrínseca da MnSOD age sobre as outras enzimas antioxidantes [Van Remmen, *et al*., 1999]. Bem como a enzima MnSOD é considerada fundamental para a manutenção das funções mitocondriais [Williams, *et al*., 1998].

```
                                                                                                                  Met Leu Ser Arg
GCGG GCGGCGCAGG AGCGGCACTC GTGGCTGTGG TGGCTTCGGC AGCGGCTTCA GCAGATCGGC GGCATCAGCG GTAGCACCAG CACTAGCAGC ATG TTG AGC CGG    106

-20                                 -10                                  1                                   10
Ala Val Cys Gly Thr Ser Arg Gln Leu Ala Pro Ala Leu Gly Tyr Leu Gly Ser Arg Gln Lys His Ser Leu Pro Asp Leu Pro Tyr Asp
GCA GTG TGC GGC ACC AGC AGG CAG CTG GCT CCG GCT TTG GGG TAT CTG GGC TCC AGG CAG AAG CAC AGC CTC CCC GAC CTG CCC TAC GAC    196

                                    20                                   30                                  40
Tyr Gly Ala Leu Glu Pro His Ile Asn Ala Gln Ile Met Gln Leu His His Ser Lys His His Ala Ala Tyr Val Asn Asn Leu Asn Val
TAC GGC GCC CTG GAA CCT CAC ATC AAC GCG CAG ATC ATG CAG CTG CAC CAC AGC AAG CAC CAC GCG GCC TAC GTG AAC AAC CTG AAC GTC    286

                                    50                                   60                                  70
Thr Glu Glu Lys Tyr Gln Glu Ala Leu Ala Lys Gly Asp Val Thr Ala Gln Thr Ala Leu Gln Pro Ala Leu Lys Phe Asn Gly Gly Gly
ACC GAG GAG AAG TAC CAG GAG GCG TTG GCC AAG GGA GAT GTT ACA GCC CAG ACA GCT CTT CAG CCT GCA CTG AAG TTC AAT GGT GGT GGT    376
                                                                    T
                                                                   Ile
                                    80                                   90                                  100
His Ile Asn His Ser Ile Phe Trp Thr Asn Leu Ser Pro Asn Gly Gly Gly Glu Pro Lys Gly Glu Leu Leu Glu Ala Ile Lys Arg Asp
CAT ATC AAT CAT AGC ATT TTC TGG ACA AAC CTC AGC CCT AAC GGT GGT GGA GAA CCC AAA GGG GAG TTG CTG GAA GCC ATC AAA CGT GAC    466

                                   110                                  120                                 130
Phe Gly Ser Phe Asp Lys Phe Lys Glu Lys Leu Thr Ala Ala Ser Val Gly Val Gln Gly Ser Gly Trp Gly Trp Leu Gly Phe Asn Lys
TTT GGT TCC TTT GAC AAG TTT AAG GAG AAG CTG ACG GCT GCA TCT GTT GGT GTC CAA GGC TCA GGT TGG GGT TGG CTT GGT TTC AAT AAG    556

                                   140                                  150                                 160
Glu Arg Gly His Leu Gln Ile Ala Ala Cys Pro Asn Gln Asp Pro Leu Gln Gly Thr Thr Gly Leu Ile Pro Leu Leu Gly Ile Asp Val
GAA CGG GGA CAC TTA CAA ATT GCT GCT TGT CCA AAT CAG GAT CCA CTG CAA GGA ACA ACA GGC CTT ATT CCA CTG CTG GGG ATT GAT GTG    646

                                   170                                  180                                 190
Trp Glu His Ala Tyr Tyr Leu Gln Tyr Lys Asn Val Arg Pro Asp Tyr Leu Lys Ala Ile Trp Asn Val Ile Asn Trp Glu Asn Val Thr
TGG GAG CAC GCT TAC TAC CTT CAG TAT AAA AAT GTC AGG CCT GAT TAT CTA AAA GCT ATT TGG AAT GTA ATC AAC TGG GAG AAT GTA ACT    736

Glu Arg Tyr Met Ala Cys Lys Lys  .
GAA AGA TAC ATG GCT TGC AAA AAG TAA ACCACGATCG TTATGCTGAG TATGTTAAGC TCTTTATGAC TGTTTTTGTA GTGGTATAGA GTACTGCAGA ATACAG    839

TAAG CTGCTCTATT GTAGCATTTC TTGATGTTGC TTAGTCACTT ATTTCATAAA CAACTTAATG TTCTGAATAA TTTCTTACTA AACATTTTGT TATTGGGCAA GTGA    947

TTGAAA ATAGTAAATG CTTTGTGTGA TTGAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAA                                        1021
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Figura 4. Sequencia deduzida de aminoácidos e nucleotídeos codificados do cDNA humano da MnSOD, adaptadas de Ho e Crapo, 1988.

O gene humano (SOD2) que codifica a MnSOD está localizado no braço longo do cromossomo 6 (6q25) [Church, *et* al., 1992] (Figura 5), possuindo cinco éxons e quatro íntrons [Wan, *et al*., 1994] . Foram localizados seis sítios polimórficos [St. Clair, 2004] (Figura 6): três na região promotora (-102; -93; -38) [Xu, *et al*., 1999]; um no éxon 2 (Ala-9Val) [Rosenblum, *et al*., 1996]; e dois no éxon 3 (I58/T; L60/F) [Borgstahl, *et al*., 1996; Hernandez-Saavedra e McCord, 2003].

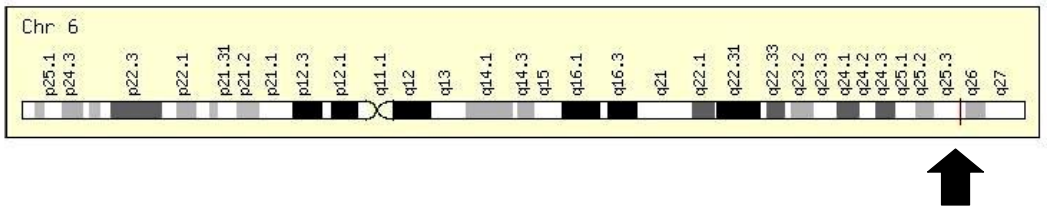


Figura 5: Posição do gene SOD2 no Cromossomo 6 humano. Extraído do site ***GeneCard*** (for protein-coding SOD2: GC06M160020).
http://www.genecards.org/cgi-bin/carddisp.pl?gene=SOD2&search=obesity+OR+diabetes.

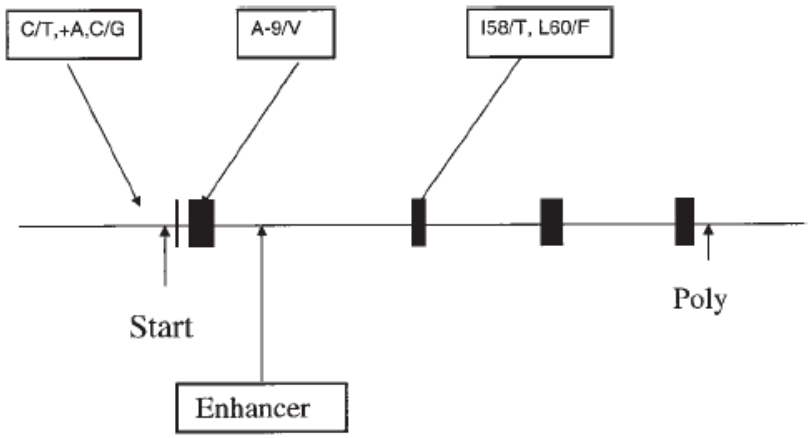


Figura 6. Organização do gene SOD2 humano e dos locais onde as variantes polimórficas foram identificadas, adaptadas de St. Clair, 2004.

No gene SOD2 foi detectado um polimorfismo de nucleotídeo único (SNP) que substitui o nucleotídeo 47 de uma citosina para uma timina (C>T), afetando o RNA mensageiro maduro, e resultando na incorporação de uma alanina (códon GCT) no lugar de uma valina (códon GTT) na região do peptídeo sinal [Rosenblum, *et al*., 1996]. O SNP intragênico resulta na alteração da conformação estrutural da enzima MnSOD: o alelo que codifica para alanina (Ala) adicionada na posição -9 da proteína madura (resíduo 16) gera uma estrutura conformacional de α-hélice ao domínio N-terminal da proteína; por outro lado, o alelo que codifica para valina (Val) gera uma estrutura conformacional β-folha no mesmo domínio, o que compromete a eficiência no transporte para MnSOD até a mitocôndria (Figura 7) [Shimoda-Matsubayashi, *et al*., 1996; Shimoda-Matsubayashi, *et al*., 1997]. Em outras palavras, a variante MnSOD -9Ala pode ser mais facilmente transportada para a mitocôndria quando comparado com a variante MnSOD -9Val.

Dados experimentais indicam que a conformação -9Ala traz uma maior atividade, devido a seu melhor transporte para a mitocôndria [Hiroi, *et al*., 1999], e que esse dimorfismo além de controlar a “importação” também regula a estabilidade do mRNA da MnSOD [Sutton, *et al*., 2005].

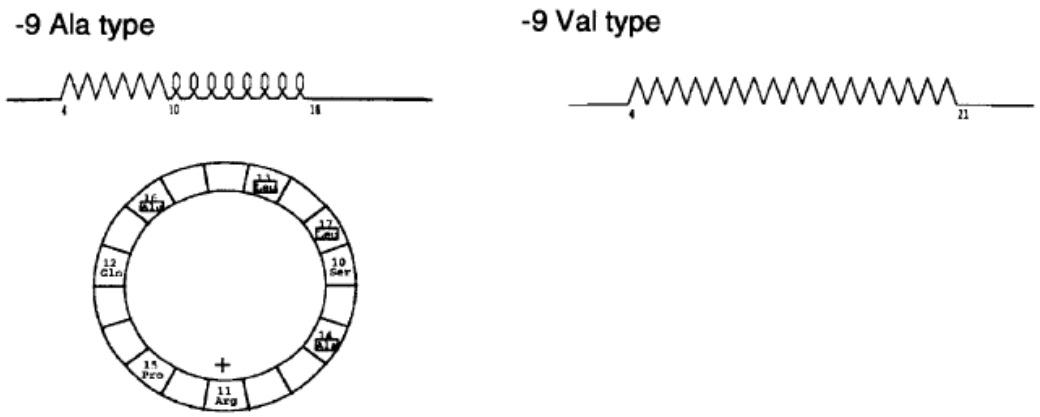


Figura 7. Diagrama esquemático da análise de predição pelo algoritmo de Chou-Fasman nas duas variantes polimórficas (-9Ala e -9Val) na região pepitídeo sinal da MnSOD humana, adaptadas de Shimoda-Matsubayashi, *et al*., 1996.

Um segundo SNP bialélico no gene SOD gera variantes protéicas na MnSOD: um alelo codifica para o resíduo 58 com uma isoleucina, e o outro alelo para o aminoácido treonina (Ile58Thr). O efeito das variantes deste SNP (no éxon 3 do gene SOD2) afeta a estabilidade e reduz a quantidade e a atividade da enzima MnSOD [Borgstahl, *et al*., 1996]. Células que super-expressam o alelo 58Ile possuem uma atividade mais elevada da MnSOD quando comparadas com células que super-expressam o alelo 58Thr [Zhang, *et al*., 1999]. Este segundo SNP tem o alelo 58Thr muito raro, podendo até não ser encontrado em muitas das populações já estudadas [Chistyakov, *et al*., 2001; Green, *et al*., 2002; Yen, *et al*., 2003; Cai, *et al*., 2004; Brans, *et al*., 2005].

***Estudos do SNP Ala-9Val em pacientes críticos de Porto Alegre, RS, Brasil***

O polimorfismo Ala-9Val do gene SOD2 que codifica a proteína MnSOD foi estudado em uma amostra de pacientes críticos da população de Porto Alegre, RS, Brasil, pela equipe do Laboratório de Genética Humana e Molecular da Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS). Foram genotipados 529 pacientes admitidos na UTI Geral do Hospital São Lucas da PUCRS (UTIG-HSL-PUCRS), sendo 356 pacientes com diagnóstico de sepse. Verificou-se que o alelo -9Ala possui uma associação

significativa com a ocorrência de choque séptico entre os pacientes sépticos (22.1% vs 77.9%, *p* = .020) [Paludo *et al*., 2013].

***Considerações Gerais***

Uma recente área das ciências da saúde é a chamada Medicina Genômica, cujo objetivo é identificar padrões genéticos que, ao serem herdados, levam o indivíduo a ser mais ou menos suscetível a desenvolver alguma doença ou característica [Guttmacher e Collins, 2002]. Entre os aproximados 25 mil genes do genoma humano [Wright *et al.*, 2001], codificadores de mais de 100 mil proteínas, já foram detectadas centenas de milhares de mutações [Lander, *et al.*, 2001; Collins, *et al.*, 2003; Burke, 2003; Collins e McKusick, 2001], entre as quais algumas podem ser relacionadas às suscetibilidades à sepse [Holmes, *et al.*, 2003; Wunderink e Waterer, 2003]. É razoável aceitar, pois, que não existem genes que determinam, estritamente, como e quando desenvolver o fenótipo e, sim, genes variantes cuja expressão favorece ou não a sua manifestação. Entre os tipos de mutações que tornam o ser humano mais suscetível, estão as variações SNP, que podem provocar alterações estruturais ou regulatórias nas diversas funções metabólicas do organismo, envolvendo, portanto as modificações fenotípicas.

A sepse é uma das principais causas de mortalidade em pacientes criticamente enfermos. Muitos mecanismos são envolvidos na fisiopatologia do choque séptico, incluindo a liberação de citocinas e a ativação de neutrófilos, monócitos e células endoteliais. Uma das rotas de dano ao metabolismo energético da célula se dá pelo acionamento da enzima poli ADP ribose polimerase (PARP) e um dos principais fatores para esta ativação é a superprodução de ERO pela mitocôndria, que promove direta ou indiretamente dano ao DNA, fragmentado-o [Fink e Evans, 2002; Massudi, *et al*., 2012; Pacher e Szabó, 2008]. Na sepse, há a liberação de Proteína de Alta Mobilidade Box 1 (HMGB-1), que age como mediador tardio na endotoxemia induzida por LPS [Wang, *et al*., 1999], ou indutor de letalidade [Yang, *et*

*al*., 2004]. HMGB-1 pode se ligar ao receptor para produtos avançados de glicação (RAGE), facilitando ativação da transcrição de NF-κB e MAPK induzindo geração de mediadores próinflamatórios em monócitos humanos e em macrófagos de roedores [Andersson, *et al*., 2000; Kokkola, *et al*., 2005]. A neutralização, através de anticorpo, para HMGB-1 e RAGE bloqueia a ativação da via de transdução de sinal da ERK½-MAP Kinase em diafragma de rato após indução de sepse atenuando a disfunção [Susa, *et al*., 2009]. Adeleção de RAGE concede proteção contra os efeitos letais do choque séptico [Liliensiek, *et al*., 2004]. As Proteínas de Choque Térmico (HSP) são um grupo altamente conservados evolutivamente de moléculas que têm um papel importante na resposta do hospedeiro a uma grande variedade de estresses, incluindo infecção, ferimentos, lesões oxidativas, hipóxia, e estresse térmico [Jaattela, *et al*., 1992; Jaattela e Wissing, 1993; De, 1999; Weiss, *et al*., 2002; Weiss, *et al*., 2006; Vinokurov, *et al*., 2012]. HSP70 modifica a resposta do hospedeiro a infecção, aumentando a resistência a diversos mediadores inflamatórios, incluindo o TNF-α e moléculas oxidantes [Satoh, *et al*., 2006; Borges, *et al*., 2012]. HSP70 também inibe a ativação do NF-kB, alterando proteínas pró-sobrevivência e expressão de citocinas [Park, *et al*., 2003; Ran, *et al*., 2004], tendo participação no mecanismo de modulação da sepse [Oberbeck, *et al*., 2007; Gelain, *et al*., 2011].

Observa-se que na sepse há um aumento intracelular de cálcio ($Ca^{2+}$) [Song, *et al*., 1993], sendo que a relação entre $Ca^{2+}$, adenosina trifosfato (ATP) e ERO está bem estabelecida: o $Ca^{2+}$ estimula o ciclo de Krebs, fazendo com que haja um aumento do fluxo de elétrons na cadeia respiratória, além de ativar vias de produção de ERO [Kumar, *et al*., 2009]. Na sepse, o $Ca^{2+}$ combinado com o aumento do NO no citoplasma [Vincent, *et al*., 2000] ou no interior da mitocôndria [Brookes, *et al*., 2004], traz como consequência a produção de Radicais Livres (RL) como o Peroxinitrito ($ONOO^-$) [Boveris, *et al*., 2002]. Essa combinação ($Ca^{2+}$ e NO agindo sinergeticamente) provoca danos à cadeia

transportadora de elétrons [Pearce, *et al*., 2001; López, *et al*., 2006], principalmente ao complexo I da cadeia transportadora de elétrons [Jekabsone, *et* al., 2003]. Estes pontos da rede de sinalização atuam contrários ao funcionamento normal da célula, promovendo o quadro séptico (Figura 8).

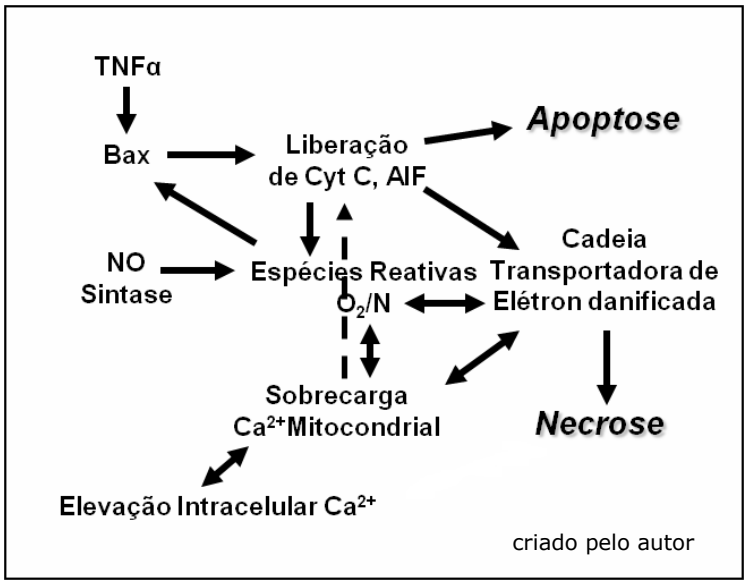


Figura 8. Esquema simplificado das vias de sinalização negativa para a homeostasia do sistema em sepse. Fator Indutor Apoptose (AIF); Citocromo C (cyt c) (criado pelo autor).

O mecanismo proposto por Szabó *et al*. (1996) sugere que o $ONOO^-$ promove dano à cadeia transportadora de elétrons e que há fragmentação do DNA ativando, assim, a PARP. Na sepse a produção de $O_2^{\cdot -}$ está aumentada [Taylor *et al*., 1995; Guidot *et al*., 1993; Andrades, *et al*., 2011], bem como a expressão do gene que codifica a enzima MnSOD [Suliman *et al*., 2003; Andrades, *et al*., 2009]. Consequentemente, devido à eficiência diferencial dos dois alelos [-9Ala (mais efetivo) e -9Val (menos efetivo)], assumiu-se que indivíduos portadores do alelo -9Ala apresentam uma maior produção de $H_2O_2$ se comparados aos portadores do alelo -9Val [Hiroi *et al.*, 1999]. O excesso de $H_2O_2$ causa dano ao DNA fragmentando-o [Kaneko *et al*., 1998; McDonald *et al*., 1993; Linley, *et al*., 2012], esses fragmentos (quebra simples e duplas) de DNA induzidos pelo $H_2O_2$ ativam a PARP [Schraufstatter *et al*., 1986; Bakondi *et al*., 2002]. O resultado final da ativação da

PARP é a formação de um equivalente bioquímico de uma NADase [Szabó *et al*.,1998]. O desfecho desse processo é uma crise energética que induzirá à morte celular [Szabó *et al*.,1997; Oliver *et al*., 1999; Wendel e Heller, 2010].

Embora que progressos foram feitos no tratamento da sepse, a taxa de mortalidade em sepse severa e choque séptico continua alta, pois o perfil da população esta em constante mudança (temos um aumento da população idosa e aumento na incidência de câncer). Por isso, torna-se óbvio a insuficiência de abordagens terapêuticas [Sales Júnior, *et al*., 2006; Henkin, *et* al., 2009; Koenig, *et al*., 2010]. Quando começamos este estudo hipotetizamos que o alelo 47C sob um estado patológico como a sepse produziria mais $H_2O_2$ que o alelo 47T, e que as peroxidases endógenas não conseguiriam eliminar este excesso de $H_2O_2$ (Figura 9).

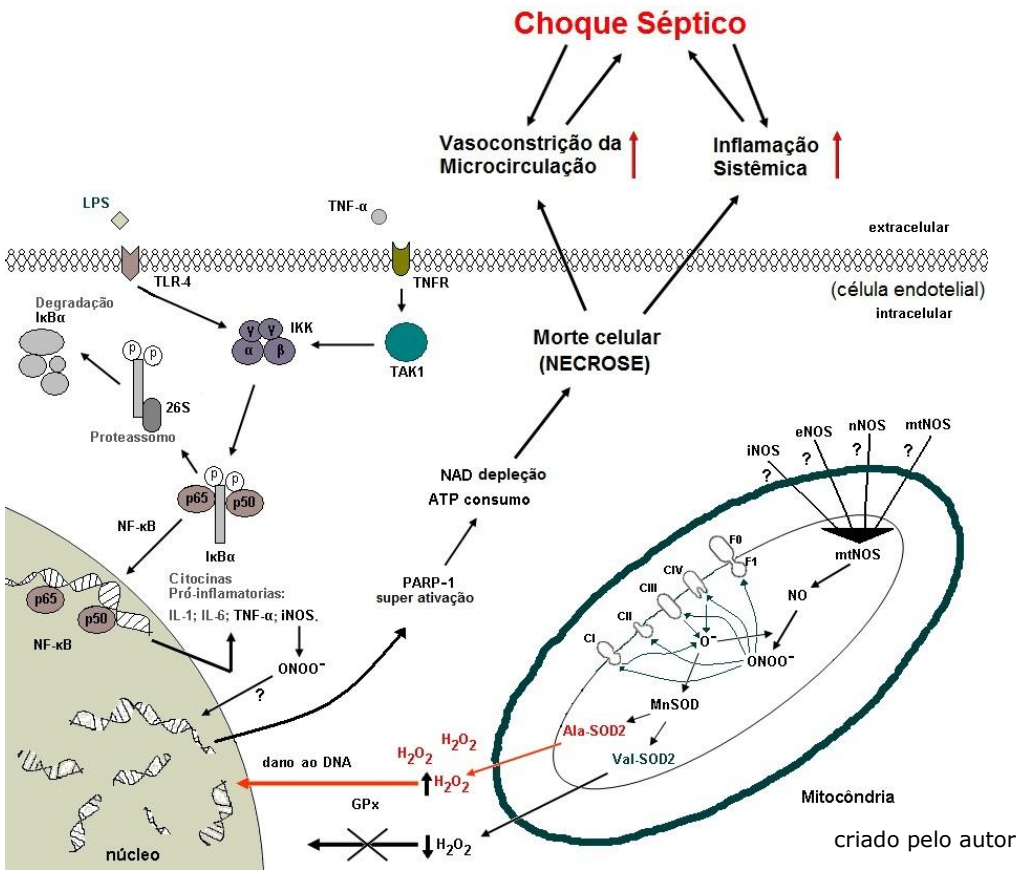


Figura 9. Hipótese do envolvimento dos alelos -9Ala ou -9Val da MnSOD na resposta inflamatória promovendo uma amplificação do dano ao DNA: ativação do NF-kB e a

síntese de fatores pró-inflamação e aumento na sensibilidade de células (particularmente células endoteliais) para radicais de oxigênio produzidos durante a inflamação tendo como consequência o dano ao DNA, depressão energética e necrose. Quinase Ativada pelo Fator de Crescimento e Diferenciação 1 (TAK1); Receptor de Fator de Necrose Tumoral (TNFR); Óxido Nítrico Síntase induzida (iNOS); Óxido Nítrico Síntase endotelial (eNOS); Óxido Nítrico Síntase neuronal (nNOS); (criado pelo autor).

Para avaliarmos essa hipótese escolhemos trabalhar com células mononucleares de sangue periférico (PBMC), uma cultura primária de fácil acesso para coleta (fato que contribui para pesquisas celulares com *background* genético). Esta cultura compõem grupos celulares que mimetizam com fidelidade a interação do sistema imune fundamental para doenças como a sepse sendo observado *in vitro* a ativação de monócitos, sua capacidade de diferenciação e apresentação de antígenos a linfócitos [Faivre, *et al.,* 2007], e liberação de citocinas [Brandtzaeg, *et al.,* 1996]. Tanto o sistema imunológico quanto o antioxidante trabalham juntos para defender o organismo contra patógenos e danos celulares característicos da sepse [Berr, *et al.,* 2007; Irshad e Chaudhuri, 2002]. Bem como escolhemos trabalhar com LPS, o maior componente estrutural e funcional da membrana externa de bactérias gram-negativas, é composto por dois componentes principais: polissacarídeo no exterior formando duas camadas de açúcar e o lipídeo A uma camada lipídica na parte interna. A porção lipídica é considerada a responsável pela maior ação antigênica do LPS [Raetz, 1990; Darveau, 1998]. A capacidade do LPS em ativar as células do hospedeiro incluindo mononucleares (monócitos e macrófagos), polimorfonucleares (neutrófilos, eosinófilos e basófilos) e endotélio, pode resultar em uma superativação levando à SIRS, ao choque séptico, e à SDMO. Por esta razão decidimos trabalhar com uma concentração definida de LPS que foi exposta a todas as amostras do que expor as mesmas a um "*pool*" de bactérias, apenas inferindo a concentração.

# OBJETIVOS

## OBJETIVO GERAL

O objetivo deste trabalho é estudar o efeito diferencial das variantes -9Ala e -9Val da MnSOD, sobre as respostas de PBMC humano (*in vitro*) desafiadas por LPS.

### *Objetivos Específicos*

- Genotipar as células para os polimorfismos Ala-9Val e Ile58Thr da MnSOD, manter culturas de PBMC humano agrupados pelos genótipos: Grupo 1 - duplo homozigotos -9Ala/-9Ala e 58Ile/58Ile; Grupo 2 - duplo homozigotos -9Val/-9Val e 58Ile/58Ile;
- Desafiar as PBMC humano com LPS (com o objetivo de se obter um ambiente com resposta inflamatória à infecção);
- Quantificar a produção de ER, e determinar os níveis das defesas antioxidantes totais não enzimáticas (TRAP) nas PBMC;
- Quantificar a atividade e imuno-conteúdo da MnSOD, CAT, GPx-1 nas PBMC;
- Quantificar indiretamente a produção de peroxinitrito através da produção de nitrotirosina, e a produção de $NO^-$ através da produção de nitrito/nitrato gerado pelas PBMC;
- Quantificar o TNF-α secretado, e os Carboximetil-lisina (CML) gerados pelas PBMC;
- Quantificar a peroxidação lipídica através da detecção dos dienos conjugados gerados nas PBMC;
- Quantificar o imuno-conteúdo da PARP, do RAGE, da HSP70, e do NFκB nas PBMC;
- Determinar os índices de dano causado ao DNA nuclear nas PBMC.

**PARTE II**

**CAPÍTULO I – Artigo Científico:**

# Participation of 47C>T SNP (Ala-9Val polymorphism) of the SOD2 gene in the intracellular environment of human peripheral blood mononuclear cells with and without lipopolysaccharides.

Mol Cell Biochem (2013)
372:127–135 DOI
10.1007/s11010-012-1453-1

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# Participation of 47C>T SNP (Ala-9Val polymorphism) of the SOD2 gene in the intracellular environment of human peripheral blood mononuclear cells with and without lipopolysaccharides

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Received: 5 May 2012 / Accepted: 5 September 2012 / Published online: 15 September 2012


**Abstract** The outcome of sepsis occurs due to influence of environmental and genetic factors besides genes variants whose expression support its outcome or not. Oxidative stress is associated to the pathogenicity of sepsis, occurring when there is a reactive species overproduction associated with inflammation. The aim of this study was to characterize the cellular redox status of human peripheral blood mononuclear cells (PBMCs) with either −9Ala (AA) or −9Val (VV) SOD2 genotypes and evaluate their response to oxidative stress induced by lipopolysaccharide (LPS). The PBMCs were isolated from the blood of 30 healthy human volunteers (15 volunteers for each allele) and the following assays were performed: antioxidant enzyme activities (superoxide dismutase; catalase; glutathione peroxidase), total radical-trapping antioxidant parameter, non-enzymatic antioxidant capacity (total antioxidant reactivity), and quantification of conjugated dienes (lipid peroxidation). At basal conditions (i.e., not stimulated by LPS), cells from 47C allele carriers showed higher activities of CAT and SOD, as well as higher TAR compared to 47T allele. However, when 47CC cells were challenged with LPS, we observed a higher shift toward a pro-oxidant state compared to 47TT cells. The CAT activity and lipid peroxidation were increased in cells with both alleles, but SOD activity increased significantly only in 47TT cells. These results demonstrate that SOD2 polymorphisms are associated with different cellular redox environments at both basal and LPS-stimulated states, and identification of this polymorphism may be important for a better understanding of pro-inflammatory conditions.

**Keywords** Sepsis · Lipopolysaccharides · Peripheral blood mononuclear cells · SOD2 Ala-9Val polymorphism · Cellular redox environment

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## Introduction

Sepsis is an important cause of admission and one of the leading causes of mortality in intensive care units (ICU), being characterized by the presence of both infection and the systemic inflammatory response syndrome (SIRS) [1, 2]. In the course of sepsis, a deficient immunologic defense may allow infection to become established; however, an excessive or poorly regulated response may harm the host through a maladaptive release of endogenously generated inflammatory compounds [3]. Septic shock represents the end of the spectrum (sepsis, several sepsis, and septic shock) of increasing inflammation and host response to an infection [3]. The release of the endotoxin lipopolysaccharide (LPS) from the cell wall of Gram-negative bacteria is generally regarded as the initiating event in the development of sepsis [4], and is well known to activate monocytes and macrophages, leading to the production of pro-inflammatory cytokines such as tumor necrosis factor-$\alpha$ (TNF-$\alpha$) and interleukin-1$\beta$ (IL-1$\beta$) [5, 6]. After activation, these cells can produce reactive oxygen species (ROS) which is addressed to kill microorganisms; however, the excess of ROS may attack cellular components causing cell

damage [7]. Antioxidant enzymes like superoxide dismutase (SOD) protect the cell from oxidative damage, presenting a potential therapeutic target for critically ill patients [8]. SOD is a family of ubiquitous metalloproteins that catalyzes the reaction of two superoxide ($O_2^-$) molecules into hydrogen peroxide ($H_2O_2$) and molecular oxygen ($O_2$) [9]. Manganese-dependent superoxide dismutase (MnSOD) (EC 1.15.1.1) exists as a tetramer and is initially synthesized containing a leader peptide, which targets this manganese-containing enzyme exclusively to the mitochondrial spaces. This enzyme is coded by a nuclear gene (SOD2) and is found in its active form within the mitochondrial matrix [10]. The protein is composed of 222 amino acids [11] and the first 24 amino acids of the primarily translated polypeptide might constitute the leader peptide for transport of the precursors to the mitochondria [12]. After synthesis in the cytosol, the inactive enzyme is transported posttranslationally into mitochondrial matrix, where it is activated upon loss of the second signal peptide [13]. The human gene encoding MnSOD is located in the long arm of human chromosome 6 (6q25) [14] and presents five exons and four introns [15]. This gene may present a change in the nucleotide 47 (C>T) sequence of DNA that will result in the incorporation of an alanine (GCT) or a valine (GTT) in the area of the signal peptide [16]. This polymorphism is predicted to generate an alteration in the conformational structure of MnSOD: the allele that encodes an alanine (Ala) added in the −9 position (residue 16) of the signal peptide-containing protein is predicted to induce an α-helical structure, while the allele encoding a valine (Val) is predicted to induce a β-sheet structure [17, 18]. This alteration (alanine to valine) is expected to affect the efficiency of the transport of MnSOD into the mitochondria, i.e., −9Ala can be more easily transported to the mitochondria when compared with −9Val [18]. The study of processing of these two types of leader signals suggests that the basal level activity of MnSOD decreases with the presence of the V allele (i.e., activity decreases from AA to AV and from AV to VV genotypes) [19], and that this dimorphism, besides controlling the efficiency of protein transport to mitochondria, also regulates the stability of the MnSOD mRNA [20].

Genomic medicine aims to identify inheritable genetic patterns in the different susceptibilities of individuals to develop some disease or characteristic. Epidemic genetic studies suggest a marked influence of genetic factors in the outcome of sepsis patients [21, 22]. The development of sepsis depends on environmental and genetic factors; expression of some gene variants was associated with different outcomes. Oxidative stress is related to the pathogenicity of sepsis, occurring when there is a reactive species overproduction associated with inflammation [23]. The aim of this study was to characterize the cellular redox status of human peripheral blood mononuclear cells (PBMCs) with either −9Ala (AA) or −9Val (VV) SOD2 genotypes and evaluate their response to oxidative stress induced by LPS.

## Materials and methods

### Subjects, design, and approval

Volunteer's participants are residents from the city of Porto Alegre, RS, Brazil (southern Brazil) which is composed of a singular genetic background: majority of inhabitants with European origin (Portuguese, Italians, Spanish, and Germans ancestry) and a small amount of individuals with African traits contributing to their genetic pool [24, 25]. To obtain the number of 30 samples, it was necessary to evaluate the genotype of 80 individuals: 47CC = 0.2, 47CT = 0.5, 47TT = 0.3 and 47C = 0.45, 47T = 0.55; Chi-square test Hardy–Weinberg equilibrium $P = 0.99$. Samples were also evaluated for another polymorphism (Ile58Thr, 173T>C) of the SOD2 gene, the allele 173C possesses smaller activity when compared to the allele 173T [26, 27] with interest in just verifying the effect of the polymorphism Ala-9Val; the heterozygote will be discarded and will just be fastened the homozygote 58Ile (173T). All human subjects signed informed consent form and the experimental protocol was approved by the Ethics Research Committee of Federal University of Rio Grande do Sul (UFRGS) protocols number 18435.

### Preparation of PBMCs

The PBMCs were isolated from the blood of healthy humans by gradient centrifugation on Ficoll-Paque™ PLUS (GE Healthcare Bio-Sciences, Uppsala, Sweden) and resuspended in RPMI 1640 (GIBCO™, Invitrogen, Grand Island, NY) supplemented with 0.28 mg/ml of gentamycin sulfate and 20 % human serum (of the own donor) at final cell density of $1.9 \times 10^6$/ml as previously described [28]. Platelet contamination of these preparations was <1 %. The viability as measured by trypan blue dye exclusion was uniformly greater than or equal to 90 %. The cells were maintained in a 5 % $CO_2$ humidified incubator at 37 °C for 18 h.

### Genotyping

Genomic DNA was extracted from leukocytes by a standard method [29]. 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 [30] for the determination of 47C>T SOD2 SNP, as well as the exon 3 segment was amplified using primers: sense 5′-AGC TGG TCC CAT TAT CTA ATA G-3′ and anti-sense 5′- TCA GTG CAG GCT GAA GAG AT-3′ (Life Technologies—Brazil Ltda. INVITROGEN Inv. São Paulo, SP, Brazil) for the determination of 173T>C SOD2 SNP [31]. 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 PCR products were digested by the *Hae*III and *Eco*RV restriction endonucleases for the Val-9Ala and Ile58Thr polymorphisms, respectively. At least 15 % of the samples were subjected to a second, independent PCR restriction fragment length-polymorphism analysis in order to confirm their genotypes.

## Experimental groups

All the 30 samples were organized in two groups: control (without LPS stimuli) and with LPS stimulation (100 ng/ml by 18 h) (*Escherichia coli* serotype 055:B5, Sigma, St. Louis, MO, USA). Fifteen samples were from 47C allele carriers, and 15 samples were from 47T allele carriers in each group. The selected healthy subjects have an average age of 26.1 years (19.8–32.4 years) and body mass index (BMI) (as stipulated by World Health Organization) of 22.3 (19.2–25.4) (Table 1), and subjects with a history of diabetes, alcohol abuse, cancer, or vitamin supplements were excluded. The cells from homozygote carriers of the 47C allele (the allele encoding alanine) were called 47CC cells; the cells from homozygote carriers of the 47T allele (the allele encoding valine) were called 47TT cells.

## Total radical-trapping antioxidant parameter (TRAP assay)

The non-enzymatic antioxidant cellular defenses were estimated by the total radical-trapping antioxidant parameter, which determines the non-enzymatic antioxidant potential of the sample, as previously described [32]. In brief, the reaction was initiated by injecting luminol and 2,2-azobis [2-methylpropionamidine]dihydrochloride (AAPH)—a free radical source that produces peroxyl radical at a constant rate—in glycine buffer (0.1 M, pH 8.6), resulting in a steady luminescent emission. PBMCs samples (10 μg of protein) were mixed in glycine buffer in their action vial and the decrease in luminescence monitored in a liquid scintillation counter for 60 min after the addition of the sample homogenates. The area under the curve obtained of the chemiluminescence values were transformed to percentage values and compared against the control values.

## Conjugated dienes

For quantification of conjugated dienes, test samples (tissue/membrane fractions) subjected to oxidative stress were treated with chloroform:methanol mixture (2:1) followed by vigorous vortexing and centrifugation at 8,000 × *g* for 10 min. The upper layer obtained was discarded

**Table 1** Demographic, clinical, and genotypic profile of donors

| Variables | All | With 47CC | With 47TT | *p* |
|---|---|---|---|---|
| Donors [n (%)] | 30 (100) | 15 (50) | 15 (50) | |
| Female [*n* (%)] | 21 (70.0) | 11 (52.4) | 10 (47.6) | $0.690^{X^2}$ |
| Age [years; mean (SD)] | 26.1 (6.3) | 25.9 (7.3) | 26.3 (5.3) | $0.954^{ST}$ |
| Weight [mean (SD)] | 65.2 (10.3) | 65.7 (10.0) | 64.7 (10.9) | $0.861^{ST}$ |
| Height [mean (SD)] | 1.71 (0.9) | 1.71 (0.06) | 1.71 (0.11) | $0.057^{ST}$ |
| BMI [mean (SD)] | 22.3 (3.1) | 22.4 (3.1) | 22.1 (3.2) | $0.915^{ST}$ |
| Smokers [n (%)] | 3 (10) | 2 (66.7) | 1 (33.3) | $0.543^{X^2}$ |
| Antidepressant [n (%)] | 5 (16.7) | 3 (60) | 2 (40) | $0.624^{X^2}$ |
| Diabetes [n (%)] | 0 (0) | 0 (0) | 0 (0) | – |
| Vitamin Supplements [n (%)] | 0 (0) | 0 (0) | 0 (0) | – |

*p* value describes a comparison between 47CC and 47TT genotype

*47CC* 47CC homozygotes, *47TT* 47TT homozygotes to 47C>T SOD2 SNP, *BMI* body mass index, *n* number, *SD* standard deviation of the mean, *ST* Student's *t* test, $X^2$ Pearson chi-square test

along with the proteins, while the lower chloroform layer was dried under a stream of nitrogen at 45 °C. The residue obtained was dissolved in cyclohexane and absorbance was taken at 233 nm against a cyclohexane. The sensitivity of this assay is up to a few nanomoles (2–3 nmol) [33].

### ELISA analyses and nitrite assay

At the end of the incubation period, the medium (supernatants) were collected, centrifuged, and frozen at −70 °C until further analysis for tumor necrosis factor-alpha (TNF-α) and nitrite. All samples were assayed in triplicate.

TNF-α protein was assessed using indirect ELISA method with a minimum level of detection ranging from 0.1 to 1,000 ηg/ml using standard curve purified protein (abcam—ab9642). In brief, the samples and standards were added and the plate was incubated overnight at 4 °C. The plate was washed four times with PBS and 0.05 % Tween 20 (Sigma, St. Louis, MO, USA). The plate was blocked with 1 % bovine serum albumin and incubated for 1 h at room temperature before washing four times with PBS and 0.05 % Tween 20. Polyclonal antibody specific for TNF-α (Sigma, St. Louis, MO, USA) was added to the wells incubated for 2 h at room temperature. After washing away, a peroxidase-conjugated secondary antibody (Cell Signaling Technology, Danvers, MA, USA) was added to the wells and incubated for 1 h at room temperature. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells. The enzyme reaction yields a blue color product that turns yellow when the stop solution is added. The color developed in proportion to the amount of TNF-α bound in the initial step. Absorbances were measured at 450 nm.

Nitrite concentrations were determined using a microplate assay based on method of Green et al. [34]. Samples supernatant (100 µl) were added to saturated solution of vanadium chloride ($VCl_3$) (400 mg), prepared in 1 M hydrogen chloride (HCl 50 ml) for reduction of nitrate to nitrite, and sodium nitrite standards in complete culture medium were mixed with 100 µl Griess reagent (1:1 0.1 % naphthyl-ethylenediamine and 1 % sulfanilamide in 5 % phosphoric acid), and then incubated for 10 min at room temperature and the nitrite content was measured by absorbance at 540 nm. Nitrite concentration in the samples was calculated with a standard curve prepared using $NaNO_2$.

### Antioxidant enzyme activities

Catalase (CAT) activity was assayed by measuring the rate of decrease of $H_2O_2$ absorbance in a spectrophotometer at 240 nm [35], and the results are expressed as U/mg protein. Superoxide dismutase enzyme activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm, as previously described [36]. Total SOD activity was assayed without KCN, and enzymatic activity of MnSOD was determined in cellular extracts by the cytochrome *c* reduction method using 1 mM KCN as previously described [37]. The difference of total and MnSOD activity was considered to represent CuZn SOD activity. To exclude the presence of nonenzymatic SOD-like activity in the homogenate, SOD activities were assayed using the boiled homogenate, and it was confirmed that the nonenzymatic activity was under the detection limit. The results are expressed as U/mg protein. To determine glutathione peroxidase (GPx) activity, the rate of NAD(P)H oxidation was measured in a spectrophotometer at 340 nm in the presence of reduced glutathione, *tert*-butyl hydroperoxide, and glutathione reductase, as previously described [38]. A ratio between SOD activity and CAT activity (SOD/CAT ratio) was applied to better understand the effect of SOD2 Ala-9Val polymorphism upon these two oxidant-detoxifying enzymes that work sequentially converting the superoxide anion to water [39]. An imbalance between their activities is thought to facilitate oxidative-dependent alterations in the cellular environment, which may culminate in oxidative stress.

### Protein determination

All the results were normalized by the protein content using the Lowry method [40].

### Statistical analysis

Results were expressed as the mean ± SEM of at least three independent experiments and each sample was performed in triplicate. Data were analyzed by Student's *t* test. When appropriate, a one-way analysis of variance (ANOVA) and individual group means compared using Tukey's multiple group comparison test was performed. To test Hardy–Weinberg equilibrium, the Chi-squared test was used, as well as to evaluate the influence of individual genotype with demographic and clinical data. Differences were considered to be significant when $p < 0.05$.

## Results

We first compared the antioxidant enzyme activities and redox parameters between 47CC and 47TT not stimulated by LPS, in order to establish if the different genotypes influence oxidative parameters under basal conditions. In untreated cells, basal SOD activity of 47CC cells was found to be higher than in 47TT cells (23.31 ± 8.48 U/mg protein

vs 19.15 ± 4.72 in 47TT, $p < 0.05$) (Fig. 1a). In this condition also, 47CC cells presented a higher basal activity for CAT (8.07 ± 2.45 U/mg protein vs 5.24 ± 1.26, $p < 0.01$) (Fig. 2a). However, we did not observe any differences in the SOD/GPx ratio, SOD/GPx + CAT ratio (data not shown) and SOD/CAT ratio between the two phenotypes (Fig. 2b). We also observed by TRAP/TAR analysis that 47CC cells possess a higher capacity of basal nonenzymatic antioxidant defenses as compared to the 47TT cells (12.7 ± 3.2 % chemiluminescence vs 20.1 ± 6.1; $p < 0.05$, respectively) (Fig. 3a, b). Besides, levels of conjugated dienes were not different between 47CC and 47TT cells, indicating that basal lipoperoxidation is not different between cells with different alleles (Fig. 3c).

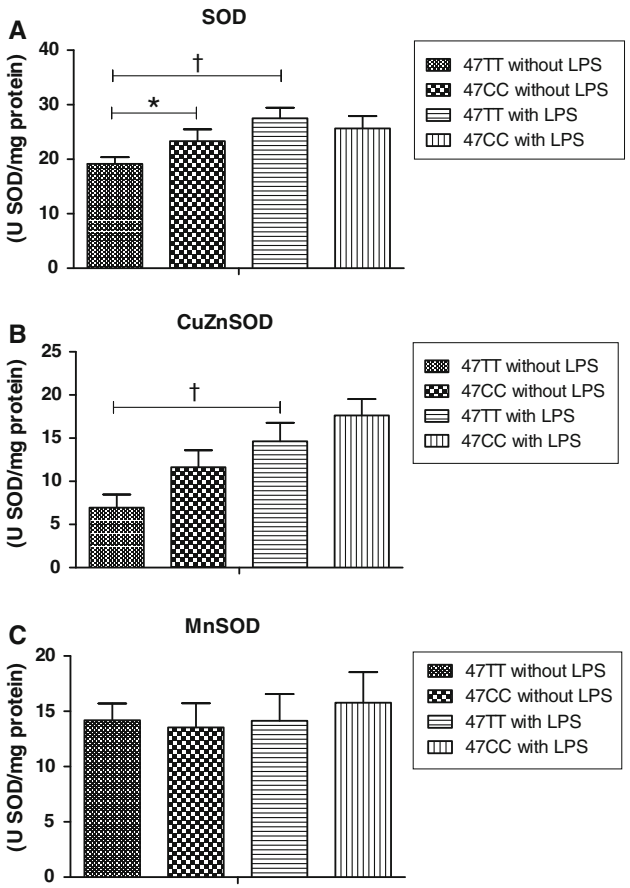


**Fig. 1** SOD activity in PBMCs separate by 47C>T SOD2 SNP challenged or not with LPS 100 ng/ml by 18 h. **a** Total SOD activity was assessed by quantifying the inhibition of superoxide-dependent adrenaline auto-oxidation in a spectrophotometer at 480 nm. The difference of total and MnSOD activity was considered to represent CuZnSOD activity (**b**). **c** MnSOD activity was determined in cellular extracts by the cytochrome *c* reduction method using 1 mM KCN. $^{*}p < 0.05$ value describes a comparison between 47CC and 47TT genotype without LPS; $^{\dagger}p < 0.05$ value describes a comparison between 47TT cells without LPS and with LPS (Student's *t* test was performed)

There was no statistical difference in GPx activity among the two cell types (43.91 ± 9.91 NADPH mM/min/mg protein vs 35.27 ± 10.98, $p = 0.660$; 47CC and 47TT cells, respectively) (Fig. 4a), as well as in the TNF-α production (0.42 ± 0.05 ηg/ml vs 0.40 ± 0.03, $p = 0.091$; 47CC and 47TT cells, respectively) (Fig. 4a, c). On the other hand, nitrite production is different one among the alleles (1.21 ± 0.54 μM vs 1.04 ± 0.30, $p < 0.01$; 47CC and 47TT cells, respectively) (Fig. 4b).

We next subjected both 47CC and 47TT to LPS stimulation as described in "Materials and methods" section and evaluated antioxidant enzyme activities and redox

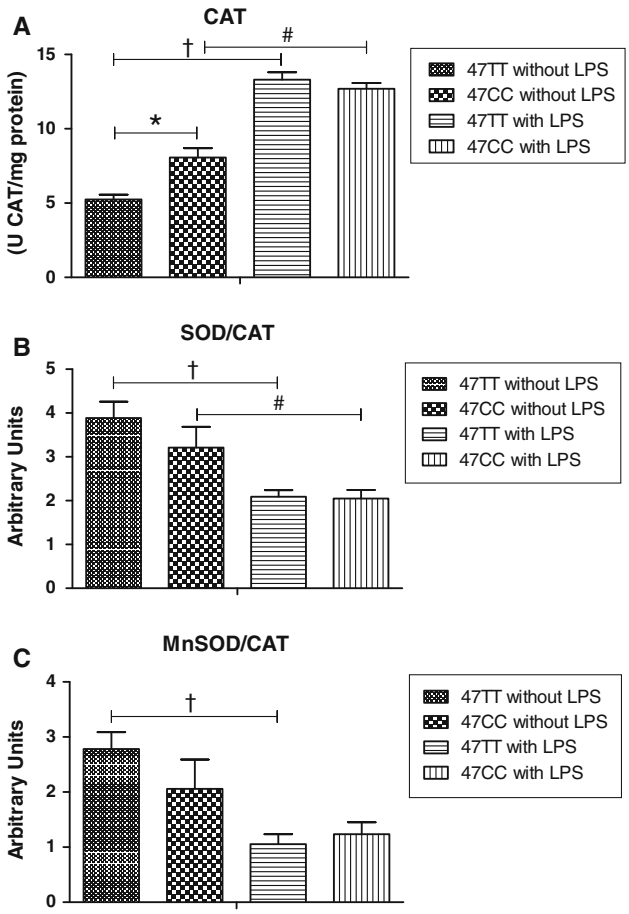


**Fig. 2** Catalase activity and ratio between SOD activity and CAT activity in PBMCs separate by 47C>T SOD2 SNP challenged or not with LPS 100 ng/ml by 18 h. **a** Catalase activity was assayed by measuring the rate of decrease in $H_2O_2$ absorbance in a spectrophotometer at 240 nm; **b** Ratio between SOD activity and CAT activity, and **c** ratio between MnSOD activity and CAT activity are applied to better understand the effect of SOD2 Ala-9Val polymorphism upon these two oxidant-detoxifying enzymes that work sequentially converting the superoxide anion to water. $^{*}p < 0.05$ value describes a comparison between 47CC and 47TT genotype without LPS; $^{\dagger}p < 0.05$ value describes a comparison between 47TT cells without LPS and with LPS; $^{\#}p < 0.05$ value describes a comparison between 47CC cells without LPS and with LPS (Student's *t* test was performed)

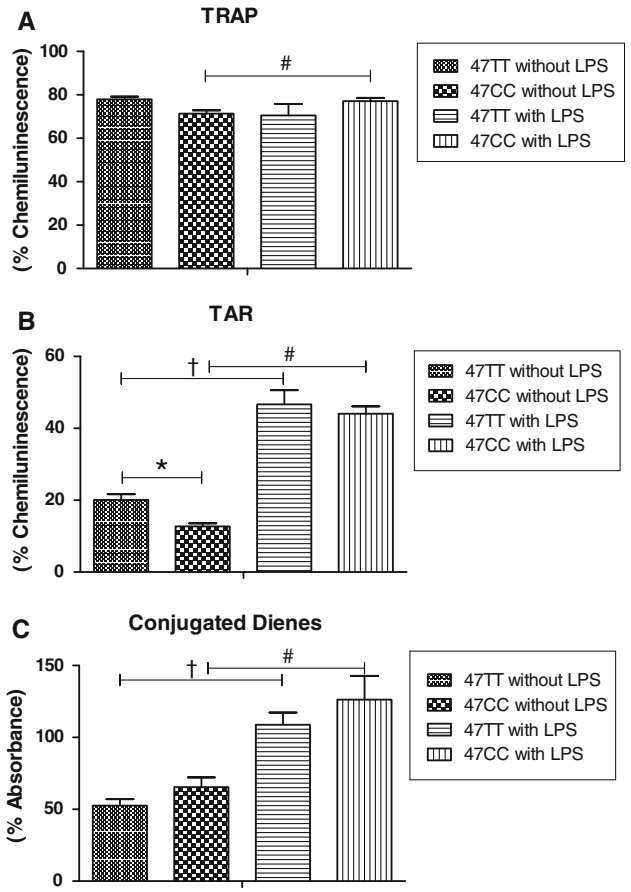


**Fig. 3** Total radical-trapping antioxidant parameter, total antioxidant reactivity, and lipid peroxidation in PBMCs separate by 47C>T SOD2 SNP challenged or not with LPS 100 ng/ml by 18 h. **a** TRAP index was measured by luminol-enhanced chemiluminescence as described in "Materials and methods" section. **b** TAR is calculations through the chemiluminescence emitted from samples at 1 min of experiment. **c** The detection of conjugated dienes is done by absorption at 233 nm. $^{*}p < 0.05$ value describes a comparison between 47CC and 47TT genotype without LPS; $^{\dagger}p < 0.05$ value describes a comparison between 47TT cells without LPS and with LPS; $^{\#}p < 0.05$ value describes a comparison between 47CC cells without LPS and with LPS (Student's *t* test was performed)

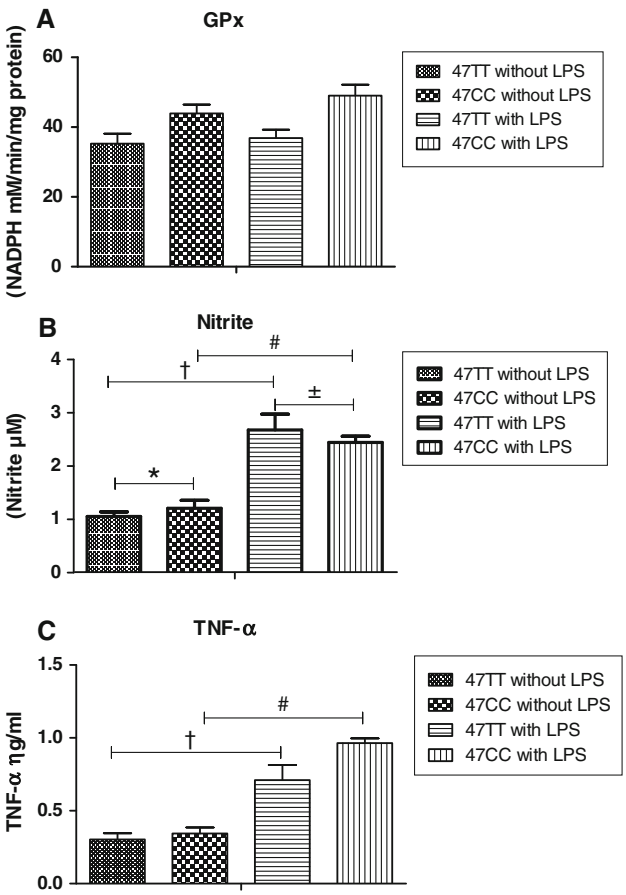


**Fig. 4** Glutathione peroxidase activity, nitrite production, and TNF-α production in PBMCs separate by 47C>T SOD2 SNP challenged or not with LPS 100 ng/ml by 18 h, at the end of the incubation period, supernatants were collected for the nitrite and TNF-α quantification. **a** GPx activity the rate of NAD(P)H oxidation was measured in a spectrophotometer at 340 nm in the presence of reduced glutathione. **b** Nitrite production (nM) was measured by the Greiss assay. **c** TNF-α release was measured by ELISA. $^{*}p < 0.05$ value describes a comparison between 47CC and 47TT genotype without LPS; $^{\dagger}p < 0.01$ value describes a comparison between 47TT cells without LPS and with LPS; $^{\#}p < 0.01$ value describes a comparison between 47CC cells without LPS and with LPS; $^{\pm}p < 0.05$ value describes a comparison between 47CC and 47TT genotype with LPS (Student's *t* test was performed)

parameters. SOD activity increased in the 47TT cells only (23.31 ± 8.48 U/mg protein vs 25.65 ± 8.81 in 47CC, $p = 0.470$; 19.15 ± 4.72 U/mg protein vs 27.52 ± 7.47 in 47TT, $p < 0.01$) (Fig. 1a) and CAT activity increased in both cell types when stimulated by LPS (8.07 ± 2.45 U/mg protein vs 12.69 ± 1.51 in 47CC, $p < 0.01$; 5.24 ± 1.26 U/mg protein vs 12.69 ± 1.51 in 47TT, $p < 0.01$) (Fig. 2a), resulting in a decrease in the SOD/CAT activity ratio (Fig. 2b). A decrease in the MnSOD/CAT ratio was observed in 47TT cells only (Fig. 2c). SOD/GPx ration and SOD/GPx + CAT ratio were not altered in both cell types (data not shown). TRAP/TAR analysis indicated that 47CC cells stimulated by LPS had a higher increase in the pro-oxidant cellular status than the 47TT cells (71.4 ± 5.9 % chemiluminescence vs 78.6 ± 4.2, and 77.1 ± 5.9 % chemiluminescence vs 75.6 ± 4.9; $p < 0.01$, respectively) (Fig. 3a, b). Moreover, both cells had equally increased lipoperoxidation induced by LPS stimulation as indicated by the increase in levels of conjugated dienes as shown in Fig. 3c. GPx activity in both 47CC and 47TT cells was not significantly altered by LPS stimulation (43.91 ± 9.91 NADPH mM/min/mg protein vs 49.02 ± 12.14 in 47CC, $p = 0.140$; 35.27 ± 10.98 NADPH mM/min/mg protein vs 36.88 ± 9.33 in 47TT, $p = 0.701$) (Fig. 4a). In nitrite production as well as difference it was

seen among the alleles in the control, it was seen in the cells challenged with LPS (2.44 ± 0.44 μM vs 2.68 ± 1.06, $p < 0.05$; 47CC and 47TT cells, respectively) (Fig. 4b), for nitrite and TNF-α production in both 47CC and 47TT cells was significant altered by LPS stimulation compared with control ($p < 0.01$) (Fig. 4b, c). But there is no difference in the TNF-α production among the challenged alleles (0.96 ± 0.12 ηg/ml vs 0.93 ± 0.15, $p = 0.233$; 47CC and 47TT cells, respectively) (Fig. 4c).

## Discussion

The 47C>T SNP may have a functional effect in the activity of MnSOD [19], but its phenotypical significances and consequences on sepsis, septic shock, organ dysfunction, or mortality are still unknown. Leukocytes, particularly the macrophages, are sensitive to oxidative stress and may have their function highly impaired under conditions of severe inflammation combined with enhanced reactive species production, such as in sepsis [41]. Reactive species, in turn, are frequently associated to the intracellular signal cascade elicited by LPS that activates the nuclear factor-κB (NF-κB) and causes the release of proinflammatory cytokines, thus establishing a chronic state of proinflammatory signaling [42].

All these signaling events are observed at several degrees in the different clinical stages of sepsis. However, sepsis outcome presents a high degree of variation which is believed to be associated to multiple environmental and genetic factors [43]. SOD2 is one of the main enzymes responsible for the so-called primary antioxidant defense response, acting directly on excess superoxide radicals derived from increased mitochondrial activity [44]. In macrophages, mitochondrial electron transport chain is greatly enhanced during the respiratory burst induced by proinflammatory stimuli [45]. Here, we compared blood mononuclear cells of 30 healthy humans volunteers separated by SOD2 47C>T SNP into two different experimental groups (15 volunteer for each allele) with the objective to investigate if there is any significative difference in the redox environment before and after macrophages presenting different SOD2 polymorphisms were challenged by LPS. Data regarding BMI, tobacco smoking, and antidepressant use were analyzed along with the results of oxidative parameters (enzymatic activity, lipid peroxidation, TAR/TRAP assay) and we found significant correlation between BMI and GPx activity after LPS stimulation and also between smokers and GPx activity in cells not treated with LPS (data not shown). It is very likely that these data reflect an influence of sample number (Table 1). However, this relationship had no influence on the results of oxidative stress parameters in PBMCs with the different alleles with or without LPS stimulation.

We observed here that although the Ala-9Val polymorphism did not significantly affect MnSOD activity (Fig. 1c), it induced a change in total SOD activity and catalase activity (Figs. 1a, 2a). Elsakka et al. [46] also reported that cells with different alleles had no significant differences among MsSOD activity; however, differently from our data, they observed a decrease in enzyme activity after the challenge with LPS. Our results suggest that the 47CC cells present a lower pro-oxidant state than the 47TT cells under basal conditions. This conclusion can be drafted from the following observations: (i) higher SOD and CAT activities; (ii) more pronounced nonenzymatic antioxidant defenses in 47CC cells, as observed with lower values of chemiluminescence (Figs. 1a, 2a, 3b); (iii) increased nitrite accumulation in allele C (Fig. 4b). However, when PBMCs are challenged by LPS, the profile of oxidant status is inverted in relationship to the basal conditions, as SOD activity was increased and the Mn-SOD/CAT ratio was decreased only in 47TT cells (Figs. 1a, 2c); the TRAP assays indicated a higher pro-oxidant status in 47CC cells (Fig. 3a). Besides, the nitrite profile also is inverted (Fig. 4b).

In some conditions of biologic stress, such as ischemia or sepsis, uncoupling of electron transport may occur due to a variety of reasons, and as a consequence, $O_2^-$ production increases [47–49]. Our data suggest that, under pro-inflammatory conditions, carriers of the 47C allele will present a higher pro-oxidant state compared to 47T allele carriers, most probably as consequence of increased $H_2O_2$ production. Hydrogen peroxide results from SOD2 detoxification activity on superoxide, being less reactive and potentially damaging to biomolecules. However, $H_2O_2$ may also favor intracellular hydroxyl radical ($OH^-$) formation through iron/copper-mediated Fenton reaction [45]. Hydroxyl radicals are highly toxic, powerfully reactive, and potent oxidizing agents, promoting oxidative stress to cells at several degrees. Thus, it is possible that an association of the 47C allele with a less favorable clinical outcome in sepsis is related to a more pronounced pro-oxidant state resulting from increased $H_2O_2$ production, especially if GPx and CAT activities are not coupled to SOD activity.

Mitochondrion is the main $O_2$ consuming and ROS-generating organelle of the cell. Suliman et al. [50] postulated that cell activation by LPS, which stimulates cytokine and ROS production, would damage mitochondria by oxidation of mtDNA. To test their hypothesis, they injected LPS intraperitoneally and evaluated several oxidative parameters in liver mitochondria. LPS injection caused a significant decrease in mtDNA copy number, and also depleted glutathione (GSH) and increased

mitochondrial lipid peroxidation. Besides, MnSOD gene expression was enhanced, thus indicating LPS treatment caused an increase in mitochondrial superoxide production, probably due to enhancement of both electron transport chain activity and uncoupling. These results reinforce the significance of SOD2 in the regulation of the redox state during pro-inflammatory stimulation.

The evaluation of critically ill patients admitted to the ICU is mainly accomplished through instruments that analyze the dysfunction of organs and systems through monitoring of their physiologic state. This approach, however, does not consider the genetic history of each patient. The genomic medicine aims to identify genetic patterns that turn the individual to be more or less susceptible to diseases [51]. A wide range of alterations (mutations) related to the susceptibilities to sepsis have been identified [22, 52]. The expression of such variants influences disease manifestation or outcome. The identification of SOD alleles in sepsis patients, in this regard, may be helpful to identify patients susceptibilities and to indicate clinical procedures more adequate for each case.

In conclusion, our results demonstrated that the Ala-9 val polymorphism of SOD2 actively participates in the regulation of cellular redox environment ,and the identification of the genotype/phenotype of patients may be a relevant step in understanding the clinical evolution of diseases with a pro-inflammatory component. Our group intends to perform further investigations in order to understand the influence of this polymorphism in other critical diseases, such as breast/prostate and lung cancer, motor neuron diseases.

**Acknowledgments** The Brazilian research funding agencies FAPERGS (PqG 1008860, PqG 1008857, ARD 11/1893-7, PRONEX 1000274) CAPES (PROCAD 066/2007), CNPq and PROPESQ-UFRGS supported this study. The authors wish to thank all volunteers participants (without you nothing would be possible).

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**CAPÍTULO II – Artigo Científico**

# Effects of 47C Allele (rs4880) of the SOD2 Gene in the Production of Intracellular Reactive Species in Peripheral Blood Mononuclear Cells with and without lipopolysaccharides induction.

Submetido para Free Radical Biology and Medicine

Elsevier Editorial System(tm) for Free Radical Biology and Medicine
Manuscript Draft

Manuscript Number:

Title: Effects of 47C Allele (rs4880) of the SOD2 Gene in the Production of Intracellular Reactive Species in Peripheral Blood Mononuclear Cells with and without lipopolysaccharides induction.

Article Type: Original Contribution

Section/Category: I have taken advantage of "Your Paper-Your Way" and my manuscript is a PDF file

Keywords: Sepsis; Lipopolysaccharides; Peripheral Blood Mononuclear Cells; SOD2 Ala-9Val polymorphism; Reactive Species.

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Abstract: Challenging of peripheral blood mononuclear cells (PBMCs) with lipopolysaccharides (LPS) associated with the cell walls of Gram-negative bacteria has been shown to activate monocytes and macrophages, leading to the production of pro-inflammatory cytokines and reactive oxygen species (ROS). These molecules are involved in the inflammatory processes associated with endotoxic shock during sepsis. Manganese superoxide dismutase (MnSOD) is an important mitochondrial antioxidant enzyme that may play a central role in the response to oxidative stress. A 47C>T single nucleotide polymorphism (SNP; ID: rs4880) has been identified in the SOD2 gene and shown to result in the production of a MnSOD protein with altered activity. The -9Val MnSOD (47T allele) is less efficient than the −9Ala version (47C allele). We have previously characterized the cellular redox status of human PBMCs expressing either -9Ala (CC) or -9Val (TT) SOD2 and analyzed the responses of these cells to oxidative stress induced by LPS. Due to the observed alterations in the activities of these antioxidant enzymes, we decided to investigate their immunocontent and analyze the production of intracellular oxidants, as well as any resulting DNA damage. PBMCs were isolated from the blood of 30 healthy human volunteers by gradient centrifugation (15 volunteers per allele). We then analyzed levels of nitrite, TNF-α, carboxymethyl lysine and nitrotyrosine and assessed production of intracellular reactive species by the 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA)-based assay. DNA damage was investigated using the comet assay, and western blots were used to analyze protein levels. Our results show that cells expressing the SOD2 47C allele quickly adapt to a more intense metabolism by upregulating cellular detoxification mechanisms. However, when these cells are stressed over a long period, they accumulate a large quantity of toxic metabolic byproducts.

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**Financial support used for the study, including any institutional departmental funds:**

The Brazilian research funding agencies FAPERGS (PqG 1008860, PqG 1008857, ARD 11/1893-7, PRONEX 1000274) CAPES (PROCAD 066/2007), CNPq and PROPESQ-UFRGS supported this work.

**Abstract**

Challenging of peripheral blood mononuclear cells (PBMCs) with lipopolysaccharides (LPS) associated with the cell walls of Gram-negative bacteria has been shown to activate monocytes and macrophages, leading to the production of pro-inflammatory cytokines and reactive oxygen species (ROS). These molecules are involved in the inflammatory processes associated with endotoxic shock during sepsis. Manganese superoxide dismutase (MnSOD) is an important mitochondrial antioxidant enzyme that may play a central role in the response to oxidative stress. A 47C>T single nucleotide polymorphism (SNP; ID: rs4880) has been identified in the *SOD2* gene and shown to result in the production of a MnSOD protein with altered activity. The -9Val MnSOD (47T allele) is less efficient than the −9Ala version (47C allele). We have previously characterized the cellular redox status of human PBMCs expressing either -9Ala (CC) or -9Val (TT) *SOD2* and analyzed the responses of these cells to oxidative stress induced by LPS. Due to the observed alterations in the activities of these antioxidant enzymes, we decided to investigate their immunocontent and analyze the production of intracellular oxidants, as well as any resulting DNA damage. PBMCs were isolated from the blood of 30 healthy human volunteers by gradient centrifugation (15 volunteers per allele). We then analyzed levels of nitrite, TNF-α, carboxymethyl lysine and nitrotyrosine and assessed production of intracellular reactive species by the 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA)-based assay. DNA damage was investigated using the comet assay, and western blots were used to analyze protein levels. Our results show that cells expressing the *SOD2* 47C allele quickly adapt to a more intense metabolism by upregulating cellular detoxification mechanisms. However, when these cells are stressed over a long period, they accumulate a large quantity of toxic metabolic byproducts.

**Key words:**

Sepsis; Lipopolysaccharides; Peripheral Blood Mononuclear Cells; *SOD2* Ala-9Val polymorphism; Reactive Species.

## Introduction

Sepsis is defined as systemic inflammation that is caused by infection, or that occurs in the presence of clinical evidence of infection [1]. The outcome of sepsis can be influenced by environmental and genetic factors, and expression of specific gene variants have been related to outcomes [2]. Release of the endotoxin lipopolysaccharide (LPS) from the cell wall of Gram-negative bacteria is generally regarded as the initiating event in the development of sepsis [3]. LPS induces well-defined, characteristic inflammatory processes. Many different mediators are involved in LPS-induced signal transduction, including protein kinase C (PKC), interleukin-6 (IL-6), mitogen-activated protein kinases (MAPKs), intercellular adhesion molecule-1 (ICAM-1), nuclear factor kappa B (NFκB), vascular cell adhesion molecule-1 (VCAM-1), plasminogen-activator inhibitor 1 (AP-1), macrophage migration inhibitory factor (MIF), and manganese superoxide dismutase (MnSOD) [4; 5]. Exposure of neutrophils and monocytes/macrophages to LPS leads to enhanced nuclear translocation of NFκB and production of pro-inflammatory cytokines [6; 7]. NFkB is an inducible transcription factor that plays an essential role in the expression of a number of genes involved in inflammatory processes, apoptosis, and intracellular redox-related processes [8; 9]. After activation, these neutrophils and monocytes/macrophages produce reactive oxygen species (ROS) which can be used to kill microorganisms or can result in changes in signal transduction and gene expression. However, excess ROS may attack cellular components, causing damage [10; 11]. Oxidative stress is related to the pathogenicity of sepsis and occurs due to inflammation-associated overproduction of ROS, leading to an imbalance between oxidants and antioxidants that can result in DNA breakage, lipid peroxidation, protein modification, membrane disruption, and mitochondrial damage [12; 13]. Mitochondrial dysfunction plays a central role in the abnormal production of biochemical compounds characteristic of sepsis [14]. Importantly, MnSOD (EC 1.15.1.1) activity has been shown to be required for maintenance of mitochondrial function [15].

MnSOD is encoded by a nuclear gene (*SOD2*), and the enzyme is activated upon loss of the second signal peptide and translocation into the mitochondrial matrix [16; 17]. The protein is composed of 222 amino acids [18] and the first 24 amino acids of the translated polypeptide probably constitute the leader peptide for transport of the precursor protein to the mitochondria [19]. After synthesis in the cytosol, the inactive enzyme is transported post-translationally

into the mitochondria [17]. Previous studies in *SOD2* knockout mice have shown that this enzyme is essential for life [20; 21] and suggest that intrinsic regulation of MnSOD expression regulates other antioxidant enzymes [22]. The human gene encoding MnSOD is located on the long arm of human chromosome 6 (6q25) [23] and contains five exons and four introns [24]. A single nucleotide polymorphism (SNP; 47C>T, ID: rs4880) has been identified that results in the incorporation of an alanine (GCT) or a valine (GTT) in the region containing the signal peptide [25]. This polymorphism is predicted to alter the conformation of MnSOD; the allele that encodes an alanine (Ala) in the -9 position (residue 16) is predicted to produce a signal peptide-containing protein that adopts an α-helical structure, while the valine (Val)-containing protein is predicted to adopt a β-sheet structure [26; 27]. This alteration (alanine to valine) is expected to affect the efficiency of the transport of MnSOD into the mitochondria, such that the -9Ala protein can be more easily transported into the mitochondria than the -9Val protein [27]. Analysis of processing of these two types of leader signals suggests that the basal activity of MnSOD decreases with the presence of the T allele (i.e., activity decreases from the CC to CT and from the CT to TT genotypes) [28]. Besides controlling the efficiency of protein transport to mitochondria, this SNP has also been shown to regulate the stability of the MnSOD mRNA [29].

Peripheral blood mononuclear cells (PBMCs), which include a mixture of immune cells, can serve as a useful model to mimic the immune system. In our previous study, we characterized the cellular redox status of human PBMCs with either -9Ala (CC) or -9Val (TT) *SOD2* genotypes and analyzed their responses to oxidative stress induced by LPS [30]. Based on the observed alterations in the activities of antioxidant enzymes, we decided to verify the immunocontent of those enzymes, and to assess the production of intracellular oxidants and potential damaging effects on the DNA.

## Materials and methods

*Subjects, design, and approval*

Volunteer participants were residents of the city of Porto Alegre, RS, Brazil (southern Brazil), which is composed of a singular genetic background. A majority of the inhabitants are of European origin (Portuguese, Italian, Spanish, and German ancestry), and a small number of individuals with African traits contribute to the genetic pool [31, 32]. To obtain 30 samples, it was necessary to evaluate the genotypes of 80 individuals, which resulted in the following

frequencies: 47CC = 0.2, 47CT = 0.5, 47TT = 0.3 and 47C = 0.45, 47T = 0.55; Chi-squared test Hardy–Weinberg equilibrium, *P* = 0.99. Samples were also evaluated for another polymorphism of the *SOD2* gene (Ile58Thr, 173T>C). The protein expressed by the 173C allele possesses decreased activity compared to that of the 173T allele [33, 34]. Because we were interest in selectively exploring the effect of the Ala-9Val polymorphism, only participants homozygous for the 58Ile (173T) allele were included in the study. All human subjects provided informed consent, and the experimental protocol was approved by the Ethics Research Committee of Federal University of Rio Grande do Sul (UFRGS; protocol number 18435).

*Preparation of PBMCs*

PBMCs were isolated from the blood of healthy humans by gradient centrifugation in Ficoll-Paque™ PLUS (GE Healthcare Bio-Sciences, Uppsala, Sweden). Isolated cells were resuspended in RPMI 1640 (GIBCO™, Invitrogen, Grand Island, NY) supplemented with 0.28 mg/mL gentamycin sulfate and 20% human serum (from the original donor) at a final density of $1.9 \times 10^6$ cells/mL as previously described [35]. Levels of platelet contamination of these preparations were <1%. To determine the viability and the population size, a Countess® automated cell counter (Invitrogen, Carlsbad, CA, USA) was used. The cells were maintained in a humidified incubator containing 5% $CO_2$ at 37°C for 18 h.

*Genotyping*

Genomic DNA was extracted from leukocytes using standard methods [36]. The polymerase chain reaction (PCR) was performed in a total volume of 25 µL and contained 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), 0.2 mM of each dNTP, 2 mM $MgCl_2$, and 10% DMSO. The exon 2 segment of the *SOD2* gene was amplified using the following 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). The underlined nucleotide represents a deliberate primer mismatch designed to introduce an artificial restriction site [37] for analysis of the 47C>T *SOD2* SNP. The exon 3 segment was amplified using the following primers: sense, 5'- AGC TGG TCC CAT TAT CTA ATA G -3' and anti-sense, 5'- TCA GTG CAG GCT GAA GAG AT -3' (Life Technologies, Brazil Ltda. INVITROGEN Inv. São Paulo, SP, Brazil) for analysis of the 173T>C *SOD2* SNP [38]. Reactions were subjected to 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, and a final extension of 7 min in a PTC-100 thermocycler (MJ Research, Inc. Watertown, MA, USA). The resulting PCR products were digested with the HaeIII and EcoRV restriction endonucleases to analyze the Val-9Ala and Ile58Thr polymorphisms, respectively. A minimum of 15% of the samples were subjected to a second, independent PCR restriction fragment length-polymorphism analysis in order to confirm their genotypes.

*Experimental groups*

The 30 samples were organized in two groups: control (without LPS stimuli) and with LPS stimulation (100 ng/mL for 18 h) (*Escherichia coli* serotype 055:B5, Sigma, St. Louis, MO, USA). Fifteen samples were from 47C allele carriers, and 15 samples were from 47T allele carriers in each group. The selected healthy subjects had an average age of 26.1 years (range, 20.1–32.1 years) and an average body mass index (BMI) (as stipulated by the World Health Organization) of 22.3 (range, 19.3–25.3) (Table 1). Subjects with a history of diabetes, alcohol abuse, cancer, or vitamin supplements were excluded. Cells from homozygous carriers of the 47C allele (the allele encoding alanine) were referred to as 47CC cells, and cells from homozygous carriers of the 47T allele (the allele encoding valine) were called 47TT cells. At the end of the incubation period, the medium (supernatant) was collected, centrifuged, and frozen at -70°C until further analysis. All samples were analyzed in triplicate, and all assays were performed three times.

*Enzyme-linked immunosorbent assay (ELISA) analyses*

ELISAs were performed to analyze changes in the content of TNF-α, 3-nitrotyrosine, and carboxymethyl lysine. In brief, the samples and standards were added to the plate (96-well MICROLON® 600 clear U bottom plate; Greiner Bio-One, Monroe USA) and incubated overnight at 4°C. The plate was then washed four times with PBS containing 0.05% Tween-20 (Sigma, St. Louis, MO, USA). The plate was blocked with 1% bovine serum albumin and incubated for 1 h at room temperature before washing four times with PBS containing 0.05% Tween-20. Polyclonal antibodies specific for TNF-α (Abcam, ab6671) and carboxymethyl lysine (Abcam, ab27684) and a monoclonal antibody specific for nitrotyrosine (Abcam, ab7048) were added to the appropriate wells, and the samples were incubated for 2 h at room temperature. After washing, a peroxidase-conjugated secondary antibody (anti-rabbit) (Cell Signaling Technology, Danvers, MA, USA) was added to the wells, and the samples were

incubated for 1 h at room temperature. Following a wash to remove any unbound antibodies, substrate solution was added to the wells. The resulting enzymatic reaction yielded a blue-colored product that turned yellow when the stop solution (chloridric acid) was added. The color developed in proportion to the amount of protein bound in the initial step. Absorbances were measured at 450 nm. TNF-α protein levels were assessed at a minimum level of detection ranging from 0.1 to 1,000 ng/mL using a standard curve based on analysis of purified protein (Abcam, ab9642).

*Nitrite assay*

Nitrite concentrations were determined using a microplate assay based on the method described by Green et al. [39]. Sample supernatants (100 µL) were added to a saturated solution of vanadium chloride ($VCl_3$) (400 mg) prepared in 1 M hydrochloric acid (HCl, 50 mL), for reduction of nitrate to nitrite. Sodium nitrite standards in complete culture medium were mixed with 100 µL Griess reagent (1:1 0.1% naphthyl-ethylenediamine and 1% sulfanilamide in 5% phosphoric acid) and then incubated for 10 min at room temperature. Nitrite content was measured by absorbance at 540 nm. Nitrite concentration in the samples was calculated based on a standard curve prepared with known concentrations of $NaNO_2$.

*Determination of intracellular reactive species (RS)*

Intracellular RS production was determined using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), a fluorescent probe that readily diffuses into cells, where it is hydrolyzed by esterases to the polar, non-fluorescent derivative 2',7'-dichlorofluorescein (DCFH), which is trapped in the cells. In the presence of RS, this compound is oxidized to the fluorescent compound DCF. Briefly, 1 x $10^5$ cells were plated on 96-well plates containing 100 µM DCFH-DA dissolved in culture medium containing 10% FBS and incubated in the dark at 37°C for 60 min before treatment was stopped to allow cellular incorporation. The cells were harvested by centrifugation (3,000 × g for 5 min), washed two times with PBS, and returned to a plate containing 200 µL PBS. Hydrogen peroxide ($H_2O_2$; 100 µM) was used as a positive control for intracellular reactive species production. DCFH-DA oxidation at 37°C was quantified based on the fluorescence emission intensity for 90 min at 5 min intervals in a fluorescence plate reader (model F2000, Hitachi Ltd., Tokyo, Japan)

with an emission wavelength set at 535 nm and an excitation wavelength set at 485 nm [40].

*Single cell gel electrophoresis: Comet assay*

The alkaline comet assay was performed as described by Singh et al. [41] with minor modifications. Briefly, the slides were prepared by mixing 20 µL culture medium with 2 x $10^4$ cells and 80 µL low melting point agarose (0.75%). The resulting mixture was added to a microscope slide coated with a layer of normal melting agarose (1.5%). After solidification, the cover slip was removed, and the slides were placed in lysis solution (2.5 M NaCl, 100 mM EDTA, and 10 mM Tris, pH 10.5, with freshly added 1% Triton X-100 and 10% DMSO) for incubation overnight at 4°C. After treatment with lysis buffer, the slides were then transferred to an electrophoresis tank containing an alkaline solution (300 mM NaOH and 1 mM EDTA). The DNA was electrophoresed for 20 min at 25 V (0.90 V/cm) and 300 mA. Afterwards, the slides were neutralized with 400 mM Tris buffer, pH 7.5, and washed three times with distilled water. Finally, the DNA was stained with SYBR Safe. Stained nuclei were analyzed by fluorescence microscopy with visual inspection in a blinded manner. All of the steps described above were carried out under red light to avoid the induction of DNA damage.

*Western Blot Analysis*

To perform immunoblot experiments, the culture media was removed and PBCM cells were lysed in Laemmli sample buffer (62.5 mM Tris_HCl, pH 6.8; 1% (w/v) SDS; 10% (v/v) glycerol). Equal amounts of proteins (30 µg) stained with Coomassie Blue were separated by SDS-PAGE, followed by transfer to a nitrocellulose membrane. The efficiency of these processes was verified by Ponceau S staining. The membrane was then blocked in Tris-buffered saline containing Tween-20 (TBST; 100 mM Tris–HCl, pH 7.5; 0.9% NaCl; 0.1% Tween-20) containing 5% albumin for 1 h at room temperature. Subsequently, the membrane was incubated overnight at 4°C with the primary antibody. Primary antibodies included anti-HSP70 (1:1000; Cell Signaling, #4872), anti-MnSOD (*SOD2*) (1:1000; Abcam, ab13533), anti-NFκ-B p65 (1:1000; Cell Signaling, #8242), anti-CAT (1:8000; Calbiochem, 219010), anti-PARP-1 (1:1000; Cell Signaling, #9532), anti-GPx-1 (1:1000; Cell Signaling, #3286), anti-RAGE (1:1000; Cell Signaling, #4679), and anti-β-Actin (1:1000; Cell Signaling, #4967). After washing in TBST, the membrane was incubated with horseradish peroxidase-coupled anti-IgG antibodies (1:5000; Cell Signaling) for 2 h at room

temperature. The membrane was washed again, and the immunoreactivity was detected by enhanced chemiluminescence. Densitometric analysis of the films was performed using IMAGE J® software. Blots were developed to be linear in the range used for densitometry. All results were expressed as a relative ratio between expression of the indicated protein and that of the β-actin internal control. Protein concentration was measured using the Bradford method [42].

*Statistical analysis*

Results were expressed as the mean ± SEM of at least three independent experiments, and each sample was analyzed in triplicate. Significant differences were analyzed by the Student's t-test. When appropriate, a one-way analysis of variance (ANOVA) and a comparison of individual group means using Tukey's multiple group comparison test were performed. To test Hardy–Weinberg equilibrium, as well as to evaluate the influence of individual genotype with demographic and clinical data, the Chi-squared test was used. Differences were considered to be significant when $p < 0.05$.

**Results**

First, a kinetics experiment was developed to determine the rate of production of reactive species (RS). The samples challenged with LPS had a lower initial fluorescence at the 0 h time point compared with samples not treated with LPS for both alleles ($p < 0.01$; Fig. 1B). This initial difference may be due to increases in the activities of some antioxidant enzymes [30], leaving few RS available to oxidize DCFH. However, individual analysis of sample fluorescence from the beginning to the end of the assay revealed an increase in fluorescence in cells treated with LPS for both alleles ($p < 0.01$; Fig. 1A). Together, these observations demonstrate that although RS is initially lower in LPS-treated cells, these cells exhibit a larger increase in RS levels over time. When $H_2O_2$ (100 μM) was added to the samples, the levels of RS in untreated cells carrying the 47C allele decreased after 60 min compared to that of cells treated with LPS. This phenomenon didn't occur in cells carrying the 47T allele, which exhibited higher levels of RS (Fig 1D). Analysis of fluorescence percentages revealed that the production of RS in the 47CC cells without LPS was higher than that of the 47TT cells without LPS. This difference was not observed in cells in the presence of LPS ($p < 0.05$; Fig. 1C).

Significant differences in nitrite/nitrate production were observed in cells carrying either allele when cells were challenged with LPS ($p < 0.01$; Fig. 2A). The same response was observed for TNF-α secretion ($p < 0.01$; Fig. 2B) and for

carboxymethyl lysine protein content ($p < 0.01$; Fig. 2D). However, analysis of 3-nitrotyrosine revealed significant differences among the alleles in the control group (optical density at 450 nm: 0.10 ± 0.01 vs. 0.12 ± 0.01 for 47TT and 47CC cells, respectively, $p < 0.05$;). There were also differences in 3-nitrotyrosine levels in 47TT cells challenged or not with LPS (0.15 ± 0.05 vs. 0.10 ± 0.01, $p < 0.01$) (Fig. 2C). To determine the optimal time to analyze the DNA damage index after challenge with LPS, we developed a dose-dependent and time-dependent response experiment (Fig. 3A) and performed a viability test (Fig. 3C). Based on results from the viability assays and the comet assay curve, we selected a time of eight hours. No significant differences were observed among the alleles, but the cells responded differently when treated or not with LPS (DNA fragmentation index: 42.5 ± 16.4 vs. 5.92 ± 5.10 in 47TT, $p < 0.01$; 46.3 ± 18.7 vs. 5.90 ± 4.54 in 47CC, $p < 0.01$; respectively; Fig. 3B).

We also evaluated HSP70, MnSOD, NFκ-B, CAT, PARP-1, GPx-1, and RAGE levels by immunoblot (Fig. 4). The samples were collected after 18 h LPS stimulation (100 ng/mL). No significant differences were observed between the groups: HSP70 - 0.85 ± 1.09 vs 1.32 ± 1.22 p= 0.322 / 0.71 ± 0.66 vs 0.53 ± 0.65 p= 0.506; MnSOD - 0.77 ± 0.68 vs 0.91 ± 0.52 p= 0.590 / 1.10 ± 0.87 vs 1.21 ± 0.70 p= 0.736; NFκ-B p65 - 0.73 ± 0.49 vs 0.72 ± 0.94 p= 0.993 / 0.75 ± 0.63 vs 0.41 ± 0.30 p= 0.107; CAT - 0.78 ± 0.38 vs 0.68 ± 0.48 p= 0.594 / 1.01 ± 0.86 vs 0.87 ± 0.54 p= 0.654; PARP-1 - 0.69 ± 0.93 vs 0.67 ± 1.10 p= 0.949 / 0.66 ± 0.90 vs 0.21 ± 0.38 p= 0.126; GPx-1 - 0.80 ± 0.78 vs 0.76 ± 0.80 p= 0.917 / 0.61 ± 0.43 vs 0.60 ± 0.43 p= 0.949; RAGE - 0.58 ± 0.59 vs 0.72 ± 0.56 p= 0.537 / 0.46 ± 0.44 vs 0.70 ± 0.54 p= 0.246 (47C allele vs 47T allele, control / LPS). HSP70 - 0.85 ± 1.09 vs 0.71 ± 0.66 p= 0.648 / 1.32 ± 1.22 vs 0.53 ± 0.65 p= 0.104; MnSOD - 0.77 ± 0.68 vs 1.10 ± 0.87 p= 0.178 / 0.91 ± 0.52 vs 1.21 ± 0.70 p= 0.174; NFκ-B p65 - 0.73 ± 0.49 vs 0.75 ± 0.63 p= 0.914 / 0.72 ± 0.94 vs 0.41 ± 0.30 p= 0.283; CAT - 0.78 ± 0.38 vs 1.01 ± 0.86 p= 0.291 / 0.68 ± 0.48 vs 0.87 ± 0.54 p= 0.078; PARP-1 - 0.69 ± 0.93 vs 0.66 ± 0.90 p= 0.897 / 0.67 ± 1.10 vs 0.21 ± 0.38 p= 0.120; GPx-1 - 0.80 ± 0.78 vs 0.61 ± 0.43 p= 0.346 / 0.76 ± 0.80 vs 0.60 ± 0.43 p= 0.434; RAGE - 0.58 ± 0.59 vs 0.46 ± 0.44 p= 0.338 / 0.72 ± 0.56 vs 0.70 ± 0.54 p= 0.850 (control vs. LPS, 47C allele / 47T allele, respectively).

**Discussion**

Fig. 1D (in which all groups were challenged with $H_2O_2$ at time 0) shows that the 47CC cells treated with LPS exhibited lower levels of RS. Furthermore,

the 47CC cells without LPS reached the same RS levels after 60 min as LPS-treated 47CC cells, which did not occur in 47TT cells. These results suggest that 47CC cells treated with LPS activated their redox capacity to a greater extent than 47TT cells in the presence or absence of LPS. These findings are consistent with the hypothesis that 47TT cells are already at their maximum redox defense capacity prior to the insult with $H_2O_2$, but that the 47CC cells without LPS do not reach maximum capacity until after 60 min of treatment with $H_2O_2$ (Fig. 1D). Nevertheless the 47CC cells without LPS produced more RS than the 47TT cells without LPS over the same period of time (Fig. 1C). In response to the oxidative stress caused by LPS-induced cellular stress response signaling and by potentiating the cellular antioxidant defense capacity, the 47C allele was shown to possess a better capacity to induce antioxidant defenses than the 47T allele. However, accumulation of relatively large amounts of ROS as a consequence of treatment with either a large dose of LPS or prolonged treatment can induce damage to cell biomolecules (proteins, lipids, and DNA). This situation can be compared to the difference between moderate physical activity and exhaustive exercise, in which the 47C allele it changes from protector to executioner, as we have previously shown [30]. These observations also explain the association between the 47C allele and septic shock (based on an observational retrospective cohort study of 529 critically ill patients) [43].

Figure 2A shows an increase in nitric oxide production in both allele groups after challenge with LPS. However, Figure 2C shows that there is no significant increase in the formation of 3-nitrotyrosine in the 47CC cells treated with LPS. Peroxynitrite ($ONOO^-$) is rapidly generated *in vivo* from nitric oxide (NO) and the superoxide anion ($O2^{\cdot-}$) [44]. $ONOO^-$ reacts with superoxide dismutase (SOD) or transition metals to form nitronium-like reactive species, which in turn nitrate tyrosine to form 3-nitrotyrosine in tissue proteins [45]. Therefore, the modified amino acid 3-nitrotyrosine is a metabolite generally associated with *in vivo* generation of peroxynitrite. Nitration of active-site tyrosines is known to compromise protein structure and function. Nitration of tyrosine has been shown to block tyrosine phosphorylation, a key event in signal transduction cascades, suggesting a role for $ONOO^-$ as a signaling molecule [46]. Thus, 47CC cells without LPS treatment (under basal conditions) exhibited tighter signaling regulation than 47TT cells, suggesting that they are in a state of readiness.

A significant increase in DNA damage was observed in PBMCs after an 8 h LPS challenge (Fig. 3B). However, it is important to mention that we also performed the assay after an 18 h LPS challenge, and no differences among the

control and LPS-treated groups were observed. Furthermore, most of the samples exhibited lower levels of DNA damage than control cells. This result suggests that LPS treatment activates repair mechanisms.

We observed a decrease in the immunocontent of cytoplasmic p-65 and PARP-1 in LPS-treated 47TT cells (Fig. 4). This result was expected, as LPS activation is thought to induce translocation of these factors into the nucleus. However, this decrease was not observed in LPS-treated 47CC cells. Several studies have already demonstrated an association between the 47C allele and diverse pathologies, such as cancer, motor neuron disease, schizophrenia, and macular degeneration [47, 48, 49, 50] and all of these pathologies are thought to be associated at least in part with redox imbalance.

**Conclusions**

In conclusion, we found that the 47C allele allows cells to quickly adapt to a more intense metabolism in the basal state, which prepares the cells for more efficient self detoxification. However, when cells carrying the 47C allele are stressed over a long period, for example with LPS, they increase the production of secondary products. A parallel can be drawn to the physical exercise situation: in spite of producing more ROS, conditioning exercise upregulates the antioxidant defense mechanism by activating cellular stress response signaling and potentiating cellular antioxidant defense capacity. However, in an exhaustive exercise situation, there is no adaptation, and the imbalance in ROS production directly damages lipids, proteins, and DNA, causing mutations or promoting tumorigenesis by activating pro-inflammatory signaling [51].

We also saw that after eight hours of LPS induction, the PBMCs exhibited a decrease in the DNA damage index. It is likely that this time period was necessary for activation of DNA repair mechanisms. In our previous study, we showed alterations in the activity of some enzymes as a result of *SOD2* allele status. However, in this study, we did not observe any differences in protein levels. Therefore, we could hypothesize that activity modulation was independent of changes in protein levels. However, changes in protein levels may have occurred at an earlier time, and these changes in expression could have downstream effects at the 18 h time point.

We have shown that the Ala-9Val polymorphism of the *SOD2* gene is associated with a worse outcome in sepsis [43] and that this polymorphism participates in regulation of the cellular redox environment [30]. Now, we show that the 47C allele promotes more efficient self-detoxification by cells. However,

when cells are stressed over a long period, they increase production of secondary byproducts associated with this process.

**Refereces**


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**Figure Legends**

**Fig. 1** Intracellular reactive species production analyzed by the DCFH-DA assay. Reactive species production was analyzed in PBMCs grouped by the 47C>T *SOD2* SNP and challenged or not with 100 ng/mL LPS for 18 hours. In (A) and (C), the percentage increase in each well was calculated according to the formula [($Ft_{90}$-$Ft_0$)/$Ft_0$ X 100], where $Ft_{90}$ = fluorescence at time 90 min and $Ft_0$ = fluorescence at time 0 min. This method of analysis has advantages over analyzing just the net change in fluorescence in that, not only did the calculated data directly reflect the percentage changes of fluorescence over time from the cells in the same well, they also effectively control for variability among wells. In (B) and (D) values are expressed as fluorescence light units/1x$10^5$ cells. In (C) and (D) $H_2O_2$ 100 µM was added to cells at the zero time point. *$p$ < 0.01 comparing 47TT cells without LPS and with LPS; #$p$ < 0.01 comparing 47CC cells without LPS and with LPS; †$p$ < 0.05 comparing 47CC and 47TT genotype without LPS. The Student's *t*-test was used for comparisons.

**Fig. 2** Nitrite production and levels of TNF-α, 3-Nitrotyrosine, and carboxymethyl lysine. PBMCs were grouped by the 47C>T *SOD2* SNP and challenged or not with 100 ng/mL LPS for 18 hours. At the end of the incubation period, the supernatants were collected for quantification of nitrite production and TNF-α levels. (A) Nitrite production (nM) was measured by the Greiss assay. (B) TNF-α release was measured by ELISA using purified protein. To analyze changes in the content of 3-nitrotyrosine (C), and carboxymethyl lysine (D) an indirect ELISA was used. Absorbance was measured at 450 nm. *$p$ < 0.01 comparing 47TT cells without LPS and with LPS; #$p$ < 0.01 comparing 47CC cells without LPS and with LPS; †$p$ < 0.05 comparing the 47CC and 47TT genotypes without LPS. The Student's *t*-test was used for comparisions.

**Fig. 3** Scoring of DNA damage and cellular viability. PBMCs were grouped by the 47C>T *SOD2* SNP and challenged or not with LPS. (A) Comet assay and (C) cell viability curves were generated by analyzing cells treated with different concentrations of LPS (0, 100, and 500 ng/mL) for different time intervals (0, 2, 4, 6, 8, 12, 16, 18 hours). (B) Extent of damage to the DNA determined by the comet assay in PBMCs grouped by the 47C>T *SOD2* SNP challenged or not with 100 ng/mL LPS for 8 hours. (D) Representative comet images showing different levels of DNA breaks in visual scoring, stained with SYBR Safe (200×). The cells were scored from 0 (no breaks observed) to 4 (maximal breaks index), according to the size and shape of the tail. *$p$ < 0.01 comparing 47TT cells without LPS and with LPS; #$p$ < 0.01 comparing 47CC cells without LPS and with LPS. A Student's *t*-test was used for comparisons.

**Fig. 4** HSP70, MnSOD, NFκ-B p65, CAT, PARP-1, GPx-1 and RAGE levels were determined by Western blot. PBMCs were grouped by the 47C>T *SOD2* SNP and challenged or not with 100 ng/mL LPS for 18 hours. Representative immunoblots with associated densitometric analyses of protein levels quantified with the IMAGE J® software are shown. Data were normalized to β-actin levels and are expressed as means ± SD for three individual experiments.

Table 1 - Demographic, clinical and genotypic profile of donors.

| Variables | All | With 47CC | With 47TT | *p* |
|---|---|---|---|---|
| Donors [n (%)] | 30 (100) | 15 (50) | 15 (50) | |
| Female [n (%)] | 22 (73.3) | 11 (50) | 11 (50) | $0.599^{X2}$ |
| Age [years; mean (SD)] | 26.1 (6.0) | 26 (7.2) | 26.2 (5.0) | $0.936^{ST}$ |
| Weight [mean (SD)] | 65.8 (10.1) | 66 (9.9) | 65.7 (10.6) | $0.936^{ST}$ |
| Height [mean (SD)] | 1.72 (0.9) | 1.71 (0.06) | 1.72 (0.11) | $0.739^{ST}$ |
| BMI [mean (SD)] | 22.3 (3.0) | 22.5 (3.1) | 22.1 (3.0) | $0.740^{ST}$ |
| Smokers [n (%)] | 3 (10) | 2 (66.7) | 1 (33.3) | $0.471^{X2}$ |
| Antidepressant [n (%)] | 5 (16.7) | 3 (60) | 2 (40) | $0.522^{X2}$ |

47CC: 47CC homozygotes and 47TT: 47TT homozygotes to 47C>T SOD2 SNP; BMI: Body Mass Index; n: number; SD: Standard Deviation of the mean; ST: Student's *t*-test; X2: Pearson Chi-Square test; *p* value describes a comparison between 47CC and 47TT genotype.

Figure 1..doc

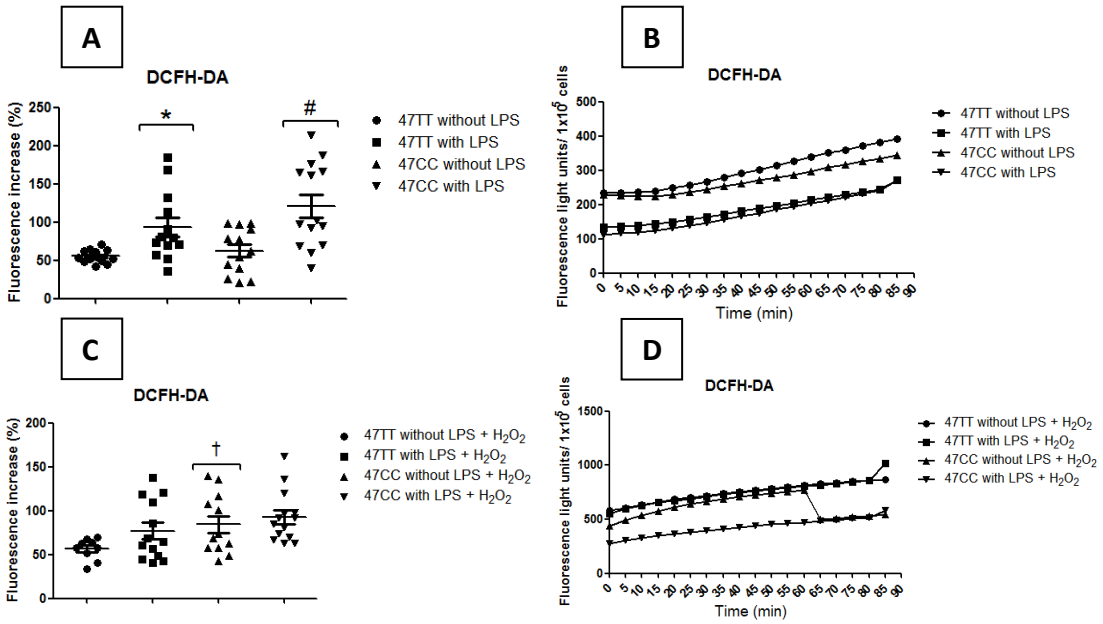

A
DCFH-DA
Fluorescence increase (%)
47TT without LPS
47TT with LPS
47CC without LPS
47CC with LPS
B
DCFH-DA
Fluorescence light units/ 1x10^6 cells
Time (min)
47TT without LPS
47TT with LPS
47CC without LPS
47CC with LPS
C
DCFH-DA
Fluorescence increase (%)
47TT without LPS + H2O2
47TT with LPS + H2O2
47CC without LPS + H2O2
47CC with LPS + H2O2
D
DCFH-DA
Fluorescence light units/ 1x10^6 cells
Time (min)
47TT without LPS + H2O2
47TT with LPS + H2O2
47CC without LPS + H2O2
47CC with LPS + H2O2

Figure 2..doc

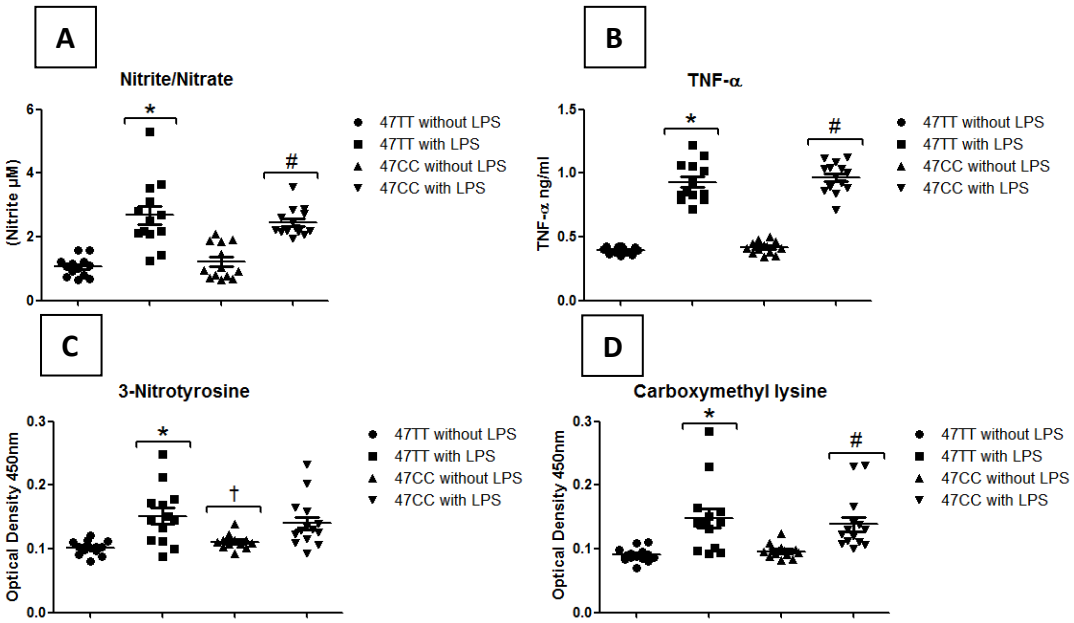

A
Nitrite/Nitrate
(Nitrite µM)
6
4
2
0
*
#
47TT without LPS
47TT with LPS
47CC without LPS
47CC with LPS
B
TNF-α
TNF-α ng/ml
1.5
1.0
0.5
0.0
*
#
47TT without LPS
47TT with LPS
47CC without LPS
47CC with LPS
C
3-Nitrotyrosine
Optical Density 450nm
0.3
0.2
0.1
0.0
*
†
47TT without LPS
47TT with LPS
47CC without LPS
47CC with LPS
D
Carboxymethyl lysine
Optical Density 450nm
0.3
0.2
0.1
0.0
*
#
47TT without LPS
47TT with LPS
47CC without LPS
47CC with LPS

Figure 3..doc

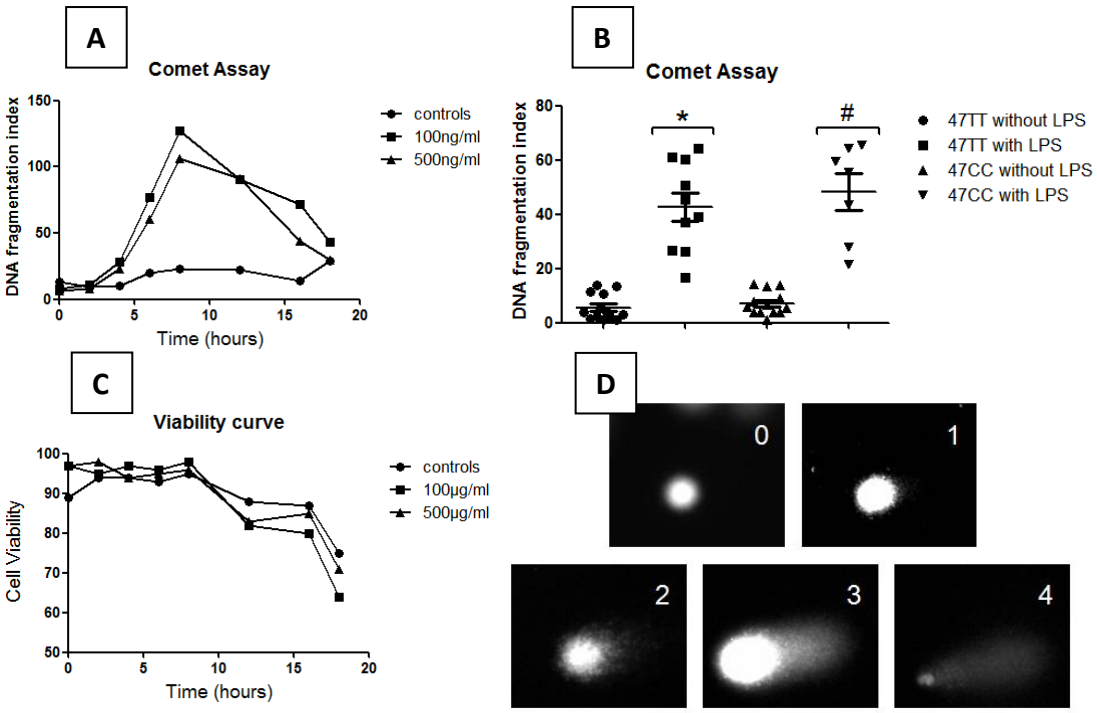
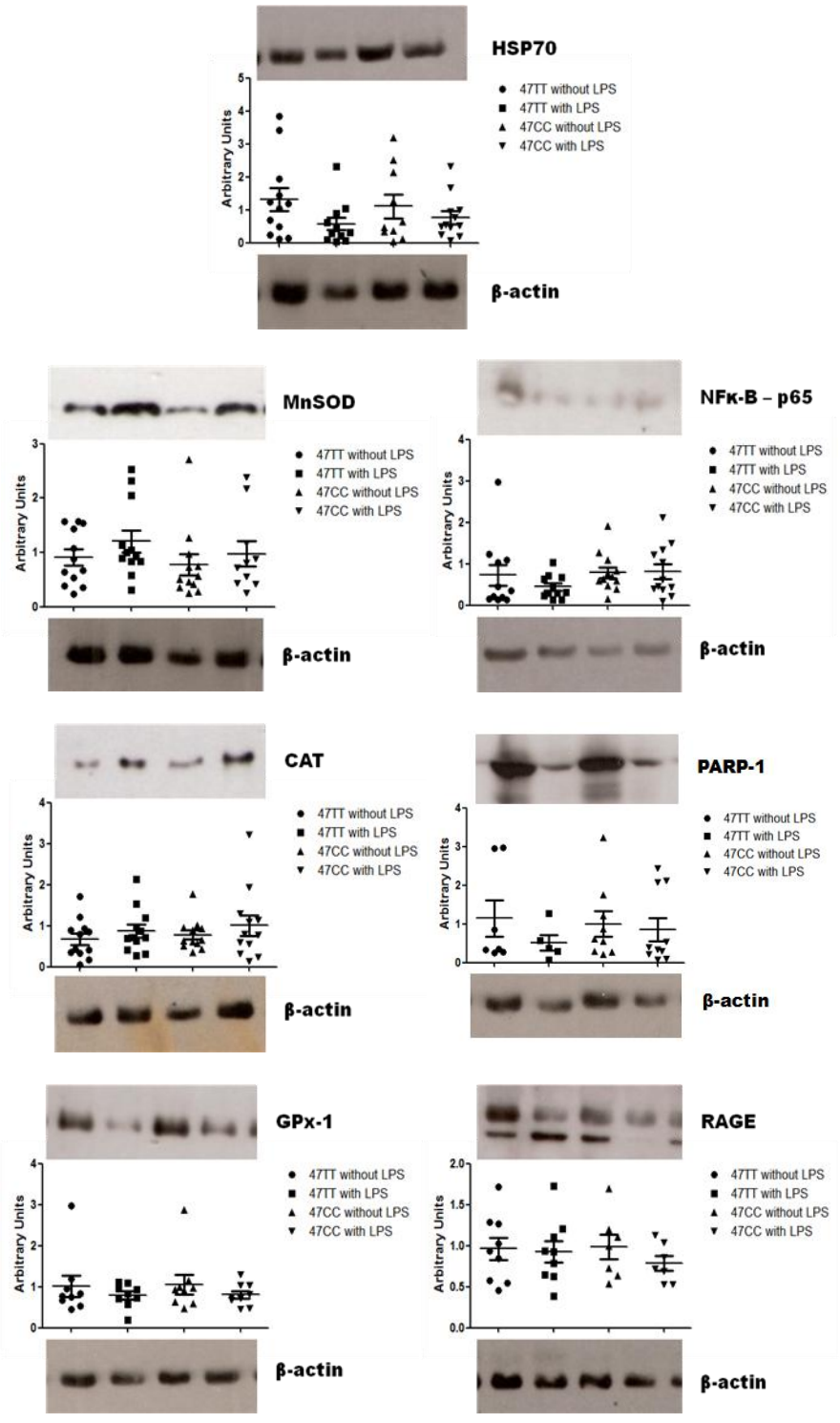

A
Comet Assay
DNA fragmentation index
Time (hours)
controls
100ng/ml
500ng/ml
B
Comet Assay
DNA fragmentation index
*
#
47TT without LPS
47TT with LPS
47CC without LPS
47CC with LPS
C
Viability curve
Cell Viability
Time (hours)
controls
100µg/ml
500µg/ml
D
0
1
2
3
4

Figure 4..doc


HSP70
47TT without LPS
47TT with LPS
47CC without LPS
47CC with LPS
Arbitrary Units
β-actin
MnSOD
NFκ-B – p65
CAT
PARP-1
GPx-1
RAGE

# PARTE III

# DISCUSSÃO

## DISCUSSÃO

A Sepse e as consequências da resposta à sepse são as causas mais comuns de mortalidade em UTI [Angus, 2010]. Vários mecanismos moleculares da inflamação e o dano celular têm sido implicadas na patogênese da sepse, choque séptico, e de SDMO, incluindo os relacionados à geração de citocinas, eicosanóides, e ERO [Macarthur, *et al*., 2000; Salvemini e Cuzzocrea, 2002]. ROS são consideradas importantes mediadores de lesão celular, contribuem para o desenvolvimento de sepse. As propriedades pró-inflamatórias de ROS incluem dano das células endoteliais, a formação de fatores quimiotáticas, recrutamento de neutrófilos, peroxidação lipídica e oxidação, danos no DNA, liberação de TNF-α e lL-1, e formação de peroxinitrito [Victor, e*t al*., 2004].

A MnSOD é uma enzima fundamental para manter controlados os níveis de oxidantes na mitocôndria como já mencionado, possui um polimorfismo funcional que inibe o transporte da enzima para a mitocôndria, consequentemente diminui os níveis atuantes da enzima. No estudo realizado com pacientes da UTIG-HSL-PUCRS, constatamos uma associação com o alelo -9Ala e o desfecho choque séptico (material anexo). Para dar continuidade no estudo, nosso próximo passo foi ver como era o ambiente celular redox das PBMC em seu estado basal separando-as pelos diferentes alelos do polimorfismo Ala-9Val. Observamos que o alelo -9Ala tinha níveis de atividade enzimática (SOD, CAT, GPx) mais elevados do que o alelo -9Val, bem como possuía uma maior quantidade (TRAP) e melhor qualidade (TAR) de defesas antioxidantes totais não enzimáticas. Porém, quando desafiávamos as células com LPS a relação se invertia. A relação SOD/CAT total diminuiu significativamente, após o LPS, para os dois alelos. Mas a relação MnSOD/CAT diminuiu significativamente apenas para o alelo -9Val, demonstrando uma modulação diferente entre os alelos. Há poucos trabalhos que demonstram a modulação da atividade das enzimas antioxidantes durante o desenvolvimento da sepse. Ritter, *et al*. (2003) mostraram uma correlação entre o aumento da atividade de SOD no plasma de ratos sépticos e mortalidade sem o aumento

proporcional na atividade de CAT. Este trabalho porém mediu a atividade apenas no plasma, ou seja a atividade verificada é da EC-SOD e não há dados na literatura que comprovem a liberação de CAT funcional para o plasma. Barichello, *et al*., (2006) mostraram a relação SOD/CAT em cerebro de ratos septicos ao longo do tempo, esta relação com o tempo é muito importante porém mais trabalhos são necessários para demonstrar em diferentes tecidos. Na literatura os resultados para a Capacidade Antioxidante Total (medido pela técnica do TRAP) e sepse são contraditórios. Trabalhos mostram uma correlação positiva com menores níveis de antioxidantes no plasma e pior desfecho na sepse [Goode, *et al*., 1995; Cowley, *et al*., 1996; Dasgupta, *et al*., 1997], e outros trabalhos mostram a correlação com maiores níveis de antioxidantes no plasma e pior desfecho na sepse [Tsai, *et al*., 2000; Chuang, *et al*., 2006; Andresen, *et al*., 2008]. E mais recentemente Gelain, *et al*., (2011) firam que dentro do grupo de pacientes septicos haviam dois grupos: com o plasma pró-oxidante; e com o plasma antioxidante, baseado nos resultados da técnica do TRAP.

Partimos então para quantificar os níveis gerados de espécies reativas (ER) utilizando uma sonda de 2',7'-diclorofluoresceína-diacetato (DCFH-DA), não encontramos diferença entre os alelos no estado basal nem quando desafiados com LPS, porém observamos que havia uma menor fluorescência das células desafiadas com LPS do que sem LPS. Possivelmente por haver menos ER disponíveis para oxidar o DCFH, pois já foi visto que na presença de LPS há um aumento nas atividades antioxidantes, mas quando analisamos a porcentagem de fluorescência produzida em 90 min visualizamos que a taxa de produção de ER é muito maior nas células com LPS. Outros trabalhos também mostraram o aumento da produção de ER, porém utilizaram polimorfonucleares do sangue de pacientes sépticos e visualizaram a fluorescência do DCF por citometria de fluxo [Martins, *et al*., 2003; Martins, *et al*., 2008]. Outro grupo observou a diminuição da produção de ER com o mesmo modelo citado anteriormente [Wenisch, *et al*., 1999]. Santos, *et al*., (2012) utilizou células mononucleares e

polimorfonucleares do sangue de pacientes sépticos e também viu aumento na produção de ER comparando com as células de voluntários.

Após acrescentarmos $H_2O_2$ em todos os grupos celulares (diferentes alelos com e sem LPS), observamos que apenas as células com o alelo -9Ala mais LPS possuíam menos fluorescência do que os outros grupos e que após 60 min de incubação com $H_2O_2$ as células com o alelo -9Ala sem LPS alcançavam os mesmos valores baixos. Ao analisarmos a taxa de fluorescência em 90 min vimos que as células com o alelo -9Ala sem LPS possuíam os mesmos valores que as células com LPS. Estes resultados mostram que provavelmente 60 min é o tempo em que as células -9Ala levaram para sinalizar o insulto gerado pelo $H_2O_2$, produzir as enzimas necessárias e colocá-las em funcionamento para diminuir o número de ER circulantes e que ao mesmo tempo neste processo, aumentavam a taxa de produção das ER.

Ao analisarmos a integridade do DNA das PBMC após 18h de cultura (com e sem LPS) uma surpresa, não havia diferença entre as células desafiadas com LPS das sem LPS, e na maioria das amostras as células com LPS possuíam menos dano do que as células sem LPS. Foi necessário realizar uma curva de tempo para definirmos o melhor momento para a análise, salientando a ativação do mecanismo de reparo ao DNA, mas nenhuma diferença foi encontrada entre os alelos. Outros trabalhos mostraram dano ao DNA em células de ratos sépticos [Kaymak, *et al*., 2008; Aydin, *et al*., 2012]. Carvalho, *et al*., (2010) procurou avaliar o prognóstico da sepse neonatal medindo o dano de DNA através do ensaio cometa, chegando ao resultado de que a técnica não foi sensível para o diagnóstico de sepse neonatal.

Na análise do imuno-conteúdo por western blot, as enzimas MnSOD e CAT apresentaram o mesmo padrão verificado na atividade, porém a enzima GPx-1 apresentou um padrão exatamente invertido. O tripeptídeo glutationa (L-g-glutamil-L-cisteinil-glicina ou GSH) é o maior regulador intracelular do sistema redox, com a ação via oxidação reversível de um grupo ativo tiol [Arrigo, 1999]. Ele pode atuar sobre

radicais livres em uma ação direta, na sua forma reduzida, o GSH inativa radicais livres doando hidrogênio para o radical, ou pode atuar indiretamente como substrato para a glutationa peroxidase (EC 1.11.1.9) [Deneke e Fanburg, 1989; Griffith, 1979]. A síntese de GSH ocorre intracelularmente a partir de glutamato, cisteína, e glicina catalisados pela γ-glutamil-cisteina-sintetase (EC 6.3.2.2) e pela glutationa-sintetase (EC 6.3.2.3) [Deneke e Fanburg, 1989]. Na cultura realizada não houve troca de meio, ou reposição destes nutrientes (glutamato, cisteína, glicina e glutamina) no período de 18h de indução com LPS e talvez isso possa explicar os resultados com a GPx-1.

HSP70 apresentou diminuição do imuno-conteúdo nas células desafiadas com LPS, resultado esperado, pois Yurinskaya, *et al.* (2009) mostraram que HSP70 tem grande participação na sepse, bem como a HSP70 é capaz de reduzir a mortalidade e modificar vários parâmetros de homeostasia e hemodinâmica de ratos sépticos [Kustanova, *et al*., 2006]. Weiss, *et al.* (2002) verificaram que aumentando a expressão de HSP70, ocorre atenuação do edema intersticial e alveolar em ratos e diminuição de acúmulo de neutrófilos no pulmão. Estes pesquisadores também observaram que o aumento na expressão de HSP70 previne a degradação proteassomal por estabilizar o complexo IKK- IκBα [Weiss, *et al.*, 2007]. A ativação do NF-κB está inibida em camundongos transgênicos e microglia que superexpressão HSP70 e isso se deve a diminuição da fosforilação da IκBα. Assim, os resultados sugestionam que HSP70 liga-se ao complexo NF-κB – IκBα evitando a fosforilação por IKK [Zheng, *et al.*, 2008].

As células -9Ala com LPS apresentaram o menor imuno-conteúdo de RAGE, bem como as células -9Val com LPS apresentaram o menor imuno-conteúdo de NFκB-p65 e de PARP-1, com a resalva de que são imuno-conteúdos citosólicos, ou seja, com LPS a tendência destas duas moléculas é de serem ativadas e se deslocarem para o núcleo. Na sepse há liberação de HMGB-1 *(*Proteína de Alta Mobilidade Box 1*)*, que age como mediador tardio na endotoxemia induzida por LPS [Wang, *et al*., 1999], ou indutor de letalidade [Yang, *et al*., 2004]. HMGB-1 pode se ligar ao receptor celular para produtos

avançados de glicação (*receptor for advanced glycation endproducts* - RAGE), facilitando ativação da transcrição de NF-κB e MAPK induzindo geração de mediadores próinflamatórios em monócitos humanos e em macrófagos de roedores [Andersson, *et al*., 2000; Kokkola, *et al*., 2005]. A neutralização, através de anticorpo, para HMGB-1 e RAGE bloqueia a ativação da via de transdução de sinal da ERK½-MAP Kinase em diafragma de rato após indução de sepse atenuando a disfunção [Susa, *et al*., 2009]. A deleção de RAGE concede proteção contra os efeitos letais do choque séptico [Liliensiek, *et al*., 2004], e Bopp *et al*. (2008) mostraram que as concentrações de RAGE solúvel (sRAGE) no plasma de pacientes sépticos eram mais elevadas do que no controle e que quanto mais grave era o estado do paciente mais elevada estava a concentração.

NFκB é um fator de transcrição central da resposta inflamatória em células imunocompetentes (tanto as periféricas como macrófagos, quanto no sistema nervoso central, como as células da glia). A ativação por estímulos inflamatórios como o TNF-α ou LPS culmina na repressão ou transcrição de vários genes, levando as células a um estado ativado pronto para responder a injúria e também para gerar proteínas que na fase anti-inflamatória induzem a finalização adequada do processo [Meffert e Baltimore, 2005; Karin e Ben-Neriah, 2000]. A regulação de NFκB é fundamental para a patologia da sepse, porém uma abordagem de inibição do NFκB, seja por inibir a ativação, ou seja, por proteólise inibindo a degradação de IkB, não pode ser apropriado para curar pacientes com sepse, a menos que possam ser administrados no início da inflamação ou no interior de compartimentos definidos [Adib-Conquy-2000].

Embora não se tenha confirmação de que algum membro da família de PARP tenha atividade enzimática de reparo, historicamente a atividade de PARP esta ligada à reparação do DNA. Isto é baseado em três principais observações: (I) dano ao DNA é o principal ativador da síntese de PAR; (II) diminuição ou inibição das PARP(s) 1-3 sensibiliza as células a agentes que danificam o DNA; (III) PARP(s) 1-3 interage

fisicamente e/ou funcionalmente com diversas proteínas de reparo de DNA [Sousa, *et al*., 2012]. PARP-1 torna-se ativado em resposta ao dano oxidativo ao DNA e esgota pools de energia celular, o que leva a uma disfunção celular em vários tecidos [Szabó, 1998]. Em choque circulatório, a PARP-1 desempenha um papel crucial tanto no desenvolvimento da disfunção cardiovascular precoce como na síndrome da resposta inflamatória sistêmica associada SDMO [Esposito e Cuzzocrea, 2009]. Inibição da atividade da PARP é protetora em vários modelos de choque circulatório [Gerö e Szabó, 2008]. Oliver, *et al*. (1999) demonstraram que PARP-1 está envolvida na regulação da via de sinalização de NFκB levando à síntese de mediadores inflamatórios, e no desenvolvimento de choque induzido por LPS endotóxico.

Embora tenham sido investigados neste estudo alguns parâmetros - dano a lipídeos, a proteínas e a DNA, além de verificar as defesas enzimáticas e não-enzimáticas do ambiente redox celular – não foi possível constatar qual o meio de ação que o polimorfismo Ala-9Val do gene SOD2 humano atua nestes parâmetros. Porém são necessários estudos adicionais com diferentes tempos de análises, pois foi constatado que este polimorfismo atua diferentemente na regulação redox.

# CONCLUSÕES

## CONCLUSÃO

Concluímos que o polimorfismo Ala-9Val do gene SOD2 humano participa na regulação do ambiente redox em PBMC e que este polimorfismo altera a produção de espécies reativas intracelulares nestas mesmas células e que isto talvez não seja fator decisivo para o desfecho clínico em pacientes sépticos, mas de grande importância para a condução do desfecho. Assim sendo, podemos concluir que há indícios para suspeitar que o gene SOD2 poderia oferecer algum prognóstico genético para doenças inflamatórias graves. O gene SOD2 parece ser um bom candidato para estudos mais aprofundados na busca de um marcador genético que, complementarmente a outras ferramentas de avaliação, possa ajudar o médico a avaliar melhor o risco do paciente. Novas investigações em variáveis moderadoras, gene-gene e interações gene-ambiente podem também ser incluídas. Em suma, os dados aqui apresentados sugerem que o polimorfismo Ala-9Val é um alvo promissor para doenças de risco genéticas, como a sepse, no qual a identificação de subconjuntos genéticos de pacientes mais suscetíveis ao pior desfecho possa ser fundamental com terapia sob medida a esses pacientes.

## CONCLUSÕES ESPECÍFICAS

- Em relação à análise de relação entre a produção de ER e o polimorfismo Ala-9Val do gene SOD2 humano o alelo -9Ala provavelmente possui mais defesas antioxidantes (devido a emitir menos fluorescência), mas produz mais ER ao longo do tempo. Este mesmo alelo no estado basal possui uma melhor quantidade (TRAP) e melhor qualidade (TAR) de defesas antioxidantes totais não enzimáticas. Entretanto após o desafio com LPS o outro alelo é que apresentou melhor quantidade (TRAP) e melhor qualidade (TAR) de defesas antioxidantes totais não enzimáticas;
- Observamos que o alelo -9Ala apresenta níveis de atividade enzimática (SOD,

CAT, GPx) mais elevados do que o alelo -9Val, porém não houve diferença no imuno- conteúdo destas enzimas após as 18h de incubação;

- Na produção de nitrotirosina, e produção de nitrito não houve diferença entre os alelos, apenas houve diferença entre os grupos com e sem LPS. O mesmo foi observado na quantificação de TNF-α e CML e peroxidação lipídica, porém nesta última houve uma tendência do alelo -9Ala a ter mais dano lipídico, tanto no basal quanto com LPS;
- Não houve diferença entre todos os grupos no imuno-conteúdo da PARP, do RAGE, da HSP70, e do NFκB após as 18h de incubação. No dano ao DNA nuclear houve diferença apenas entre as células com e sem LPS.

# PERSPECTIVAS

## PERSPECTIVAS

Com o intuito de especificar melhor o mecanismo de atuação do polimorfismo em PBMC estressadas com LPS.

As principais perspectivas do seguimento deste trabalho são:

1. Desafiar as PBMCs humano (Grupo 1 e Grupo 2; ver objetivos específicos) com LPS na concentração de 1µg/mL e verificar o imuno-conteúdo da PARP, do RAGE, da HSP70, e do NFκB após duas e seis horas de indução;
2. Avaliar a porcentagem de células com marcação para apoptose e células necroticas/mortas através dos marcadores Anexina V e Iodeto de Propídio;
3. Avaliar a integridade das mitocôndrias das PBMC humano dos Grupos 1 e 2 após o desafio com LPS através da técnica de *Swelling Mitochondrial*;
4. Medir a atividade dos complexos enzimáticos da cadeia respiratória das PBMC humano dos Grupos 1 e 2 após o desafio com LPS;
5. Avaliar todos estes ensaios acrescentando aos grupos o inibidor da Óxido Nítrico Sintase NG-nitro-L-arginina metil éster (L-NAME).

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## ANEXOS

Anexo 1: Lista de figures;

Anexo 2: Termo de Consentimento Livre e Esclarecido;

Anexo 3: Questionário de Coleta de Sangue;

Anexo 4: Artigo Publicado: Higher frequency of septic shock in septic patients with the 47C allele (rs4880) of the SOD2 gene. Gene. 2013; 517(1):106-11. doi: 10.1016/j.gene. 2012.10.078.

## LISTA DE FIGURAS

**TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO**



**Título da pesquisa:**
EFEITOS CELULARES DA VARIANTE POLIMÓRFICA Ala-9Val DA MnSOD HUMANA, DA MELATONINA, E DO ETIL PIRUVATO SOBRE O ESTRESSE OXIDATIVO DURANTE O PROCESSO INFECCIOSO: ESTUDO *in vitro.*

**O que queremos pesquisar (objetivo da pesquisa):**

Com nossa pesquisa queremos conhecer como funcionam as células que herdam diferentes versões genéticas. Avaliar as funções de um gene chamado SOD2, e se as substâncias Melatonina e Etil Piruvato trazem alguma melhora para essas células quando prejudicadas pelos Lipopolisacarídeos (moléculas da superfície das bactérias).

**Como faremos isso (procedimentos a serem utilizados):**

Vamos coletar células mononucleares de sangue periférico humano, cultivar essas células no laboratório separando-as de acordo com as diferentes versões do gene SOD2 (chamados alelos para -9Ala/-9Val e 58Ile/58Thr). Após, realizaremos ensaios com as células na intenção de descobrir se elas se comportam de forma similar ou diferente diante de certos tratamentos.

**De onde virão as células (fonte do material biológico):**

Numa primeira etapa será realizada coleta de sangue (procedimento estéril e descartável) de cerca de 01 mililitros para constatação de qual versões do gene SOD2 o(a) voluntário(a) possui. Após confirmação da versão de interesse (genótipo) para a pesquisa, será realizada uma segunda coleta de sangue (etapa 2) na qual serão coletados 20 mililitros de sangue (procedimento estéril e descartável) para separação e cultivo das células.

**Os desconfortos ou riscos esperados para quem doar o sangue:**

Inerente a doação do sangue está a dor relacionada a punção venosa e também existe a possibilidade de hematoma local. Todo material utilizado será estéril e descartável, desta maneira será evitado qualquer risco ao doador.

**Garantia de resposta a qualquer pergunta;**

O(A) doador(a) ou qualquer pessoa envolvida com o(a) mesmo(a) pode se sentir à vontade para fazer qualquer pergunta sobre a pesquisa, ou sobre os procedimentos, a fim de esclarecer suas dúvidas. Os responsáveis pela pesquisa garantidamente responderão.

**Compromisso com informação atualizada do estudo:**

Toda e qualquer informação atualizada sobre este estudo estará à disposição de todos os que participam do mesmo. Os resultados deste estudo serão comunicados aos participantes da pesquisa, sempre que solicitados.

| | |
|---|---|
| Rubrica do(a) doador(a) | Rubrica do Pesquisador |

**Os benefícios que se pode obter:**

Neste momento, nem o(a) doador(a), nem ninguém receberá algum benefício direto em decorrência desta pesquisa. No entanto, haverá benefícios para a ciência, pois os dados das células irão ajudar a entender as doenças humanas complexas. Mesmo assim, no futuro, caso alguma informação considerada importante seja identificada no material biológico analisado em decorrência desta pesquisa, os pesquisadores buscarão o(a) doador(a) para oferecer-lhe acesso a tal informação.

**Liberdade de abandonar a pesquisa sem prejuízo para si:**

Em qualquer momento, se o(a) doador(a) achar que não quer mais que se use o material que lhe foi coletado, é só avisar que automaticamente será atendido(a). Não há a necessidade de que se apresentem justificativas. Nem o(a) doador(a) nem qualquer outra pessoa vinculado a ele(a) sofrerá algum tipo de prejuízo. Apenas pedimos que informem aos pesquisadores sua decisão de abandonar a pesquisa. Neste momento, será desconsiderado o consentimento dado no início.

**Garantia de Privacidade:**

Nós asseguramos que cuidaremos de manter a dignidade e a privacidade dos participantes da pesquisa, em relação a qualquer dado utilizado. Os dados dos participantes estarão protegidos pelos pesquisadores responsáveis contra qualquer tipo de uso que não os previstos na investigação. Além disto, os dados receberão um número pelo qual serão identificados, garantindo assim, também, o anonimato dos participantes. A identidade dos participantes será desconhecida pela equipe do trabalho de pesquisa.

**Permissão para uso da amostra de DNA e outros dados em estudos futuros:**

A partir das mesmas células poderão ser futuramente feitos outros estudos genéticos. Para isso, o participante poderá permitir que seu material seja usado de novo em estudos complementares. Mesmo que esta permissão seja dada agora, estudos futuros só poderão ser realizados se forem aprovados antes por um Comitê de Ética em Pesquisa.

| | |
|---|---|
| Rubrica do(a) doador(a) | Rubrica do Pesquisador |

**TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO**



Eu, _____ (*preencher com nome do(a) doador(a)*) fui informado(a) dos objetivos da pesquisa de maneira clara e detalhada. Recebi informação sobre os procedimentos do estudo e esclareci minhas dúvidas. Sei que em qualquer momento poderei solicitar novas informações e modificar minha decisão se assim eu o desejar. A equipe do Drº. José Cláudio Fonseca Moreira me certificou de que todos os dados referentes ao material doado serão confidenciais. Entendo também que tenho a liberdade de retirar meu consentimento de participação na pesquisa se eu quiser. Assim, dou consentimento para que as células do meu sangue sejam coletadas e estudadas.

Também permito que esse material seja usado em pesquisas futuras, sem a necessidade de nova consulta, desde que os novos estudos sejam devidamente aprovados por um Comitê de Ética em Pesquisa e estejam sob responsabilidade do mesmo Pesquisador Coordenador. Fui informado que, também nesta situação, caso alguma informação considerada importante seja identificada em decorrência da pesquisa, os pesquisadores farão contato a fim de oferecer acesso a tal informação.

Se me ocorrerem novas perguntas sobre este estudo, posso telefonar ao Msc. Francis Jackson de Oliveira Paludo nos telefones (51) 97842671 ou (51) 3308 5578. Para qualquer pergunta sobre os meus direitos como participante deste estudo, ou se penso que fui prejudicado(a) pela minha participação, posso fazer contato também com ao Drº. José Cláudio Fonseca Moreira nos telefones (51) 99852696 ou (51) 3308 5577 e os responsáveis pelo Comitê de Ética em Pesquisa da UFRGS no telefone (51) 3308 3629. As ligações para os celulares poderão ser realizadas a cobrar.

Declaro que recebi cópia do presente Termo de Consentimento.

| | | |
|---|---|---|
| **Assinatura do(a) doador(a)** | **Nome do(a) doador(a)** | **____ / ____ / 20____**<br>**Data** |
| **Assinatura do Pesquisador** | **Nome do Pesquisador** | **____ / ____ / 20____**<br>**Data** |

Na impossibilidade de autonomia para a leitura deste Termo de Consentimento Livre e Esclarecido, o mesmo foi lido para _____ (*preencher com nome do(a) doador(a)*) pelo pesquisador _____ (*preencher com nome do pesquisador*) enquanto eu estava presente como testemunha.

| | | |
|---|---|---|
| **Assinatura da Testemunha** | **Nome da Testemunha** | **___ / ____ / 20____**<br>**Data** |

Nº da amostra

**Universidade Federal do Rio Grande do Sul**

**Instituto de Ciências Básicas da Saúde**

**Programa de Pós-Graduação em Ciências Biológicas - Bioquímica**

**PROJETO DE PESQUISA:** EFEITOS CELULARES DA VARIANTE POLIMÓRFICA Ala-9Val DA MnSOD HUMANA, DA MELATONINA, E DO ETIL PIRUVATO SOBRE O ESTRESSE OXIDATIVO DURANTE O PROCESSO INFECCIOSO: ESTUDO *in vitro.*

**QUESTIONÁRIO PARA COLETA DE DADOS**

Nome:

Qual sua idade?_____

Qual seu peso/altura?_____

É diabético(a)? ( ) Não. ( ) Sim. Qual tipo?_____

É fumante? ( ) Não. ( ) Sim. Quanto tempo?_____

Faz uso de medicamento antidepressivo? ( ) Não. ( ) Sim. Qual? _____

Faz suplementação alimentar com vitaminas? ( ) Não. ( ) Sim.

Esteve resfriado nos últimos sete (07) dias? ( ) Não. ( ) Sim. Quando?_____

Esteve gripado nos últimos sete (07) dias? ( ) Não. ( ) Sim. Quando?_____

Esteve com febre nos últimos sete (07) dias? ( ) Não. ( ) Sim. Quando?_____

Tomou medicamento com ácido acetilsalicílico (Aspirina, AAS, Buferin, Doril, Melhoral, Cibalena, Doloxene-A, etc) nos últimos três (03) dias? ( ) Não. ( ) Sim. Quando?_____

Tomou antibiótico nos últimos sete (07) dias? ( ) Não. ( ) Sim. Quando?_____

Tomou anti-inflamatório nos últimos sete (07) dias? ( ) Não. ( ) Sim. Quando?_____

Gene 517 (2013) 106–111

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Methods Paper

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

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ARTICLE INFO

*Article history:*
Accepted 31 October 2012
Available online 8 November 2012

*Keywords:*
Critical illness
Sepsis
Septic shock
47C>T *SOD2* SNP
*SOD2* Ala-9Val polymorphism

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.

☆ Financial support used for the study, including any institutional departmental funds: This study was financed by Edital FAPERGS/MS/CNPq/SESRS 002/2009 Pesquisa para o SUS: GESTAO COMPARTILHADA EM SAUDE PPSUS — 2008/2009.

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http://dx.doi.org/10.1016/j.gene.2012.10.078

(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 $X^2$ | $\beta$ | S.E. ($\beta$) | 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 $X^2$ | $\beta$ | S.E. ($\beta$) | 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; $\beta$: 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.

## Acknowledgments

We thank CAS Ferreira, CL Dornelles, and FB Nunes for their suggestions and P Graebin, TJ Borges, CO Alminhana, and HS Thurow for technical assistance. This study was financed by the Fundação de Amparo a Pesquisa do Estado do rio Grande do Sul - FAPERGS and Conselho Nacional de Desenvolvimento Científico e Tecnológico — CNPq (process # 09/0039-3/PPSUS), the Programa de Bolsa Pesquisa para Alunos da Graduação — Edital BPA PUCRS 2007–2008, and the Faculdade de Biociências, PUCRS. The study is part of the Masters' Degree dissertation of the first author who had a fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior — CAPES, Brazil.

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