Pontifícia Universidade Católica do Rio Grande do Sul
Faculdade de Biociências
Programa de Pós-Graduação em Biologia Celular e Molecular

FRANCIS JACKSON DE OLIVEIRA PALUDO

ESTUDO DA VARIANTE POLIMÓRFICA 47C>T (Ala-9Val) DO GENE QUE CODIFICA PARA A SUPERÓXIDO DISMUTASE DEPENDENTE DE MANGANÊS (MnSOD) EM PACIENTES EM CONDIÇÕES CRÍTICAS DE SAÚDE

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Dissertação apresentada como requisito para obtenção do grau de Mestre pelo Programa de Pós-Graduação em Biologia Celular e Molecular da Pontifícia Universidade Católica do Rio Grande do Sul

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Dedico esta dissertação ao motivo que me faz sorrir todos os dias, que me faz ter forças para avançar não importando a dificuldade, que me faz amar a minha vida e a preenche com a infinita felicidade.

Enfim, dedico esta dissertação a minha esposa Kátia e minha filha Mariana.

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RESUMO

Para avaliar as duas versões do gene SOD2 que codifica para a proteína superóxido dismutase dependente de manganês na sepse, nós determinamos a freqüência do polimorfismo de nucleotídeo simples (SNP) 47C>T em pacientes criticamente doentes com sepse (n=356) e sem sepse (n=173), e também investigamos as freqüências genotípicas nos desfechos adversos à sepse (choque séptico e mortalidade) no grupo de pacientes sépticos. Nós comparamos os portadores do alelo 47C (genótipos 47CC+47CT) com homozigotos 47TT e demonstramos uma associação estatisticamente significativa entre os portadores do alelo 47C e a ocorrência de choque séptico no subgrupo de pacientes sépticos (p=0.025). Na análise ajustada de regressão logística binária, incorporando o SNP 47C>T e os principais preditores clínicos, nós mostramos que somente o escore SOFA [p<0.001, OR=9.107 (95%CI=5.319-15.592)] e o alelo 47C [p=0.011, OR=2.125 (95%CI=1.190-3.794)] foram significativamente associados com o desfecho de choque séptico. Nossos resultados e nossa hipótese sugerem que a mais alta freqüência de portadores do alelo 47C em pacientes sépticos com desfecho desfavorável é provavelmente explicada por um efeito no estresse celular.

ABSTRACT

In order to analyze the effect of the two different versions of the manganese superoxide dismutase gene (SOD2) on sepsis, we determined the 47C>T single nucleotide polymorphism (SNP) frequencies in critically ill patients with (n=356) and without sepsis (n=173), and also investigated the genotype frequencies in adverse outcomes in the septic patient's subgroup. We compared the 47C allele carriers (47CC+47CT genotypes) with 47TT homozygotes and we demonstrated a significant association between 47C allele carriers and septic shock in septic patients (p=0.025). With an adjusted binary logistic regression, incorporating 47C>T SNP and the main clinical predictors, we showed that only SOFA score [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. Our results and our hypothesis suggest that the higher 47C allele carrier frequency in septic patients with negative outcome is possibly explained by an effect on cellular stress.

LISTA DE ILUSTRAÇÕES

LISTA DE SIGLAS

Ala - Alanina
bpm - batimentos por minuto
CD14 - *Cluster of Differentiation*
cels/mm^3 - células por milímetros cúbico
CEP-PUCRS - Comite de Ética em Pesquisa da Pontifícia Universidade Católica do Rio Grande do Sul
CuZnSOD - Superóxido Dismutase Cobre/Zinco dependente
DMSO - Dimetilsulfóxido
DNA - Ácido Desoxirribonucléico
dNTPs - Desoxirribonucleodídeos Fosfatados
EAOs - Enzimas Antioxidantes
EC-SOD - Superóxido Dismutase Extracelular
EDTA - Ácido Tetraacético de Etilenodiamina
EROs - Espécies Reativas de Oxigênio
GPx - Glutationa Peroxidase
HIV - Vírus da imunodeficiência Humana
HSL - Hospital São Lucas
H_2O_2 - Peróxido de Hidrogênio
IFN- γ - Intérferon Gamma
IL - Interleucina
irpm - impulso respiratório por minuto
LPS - lipopolisacarídeos
mmHg - milímetros de mercúrio
MnSOD - Superóxido Dismutase dependente de Manganês
MODS - Síndrome da Disfunção Orgânica Múltipla
MOF - Falência de Múltiplos Órgãos
mRNA - Ácido Ribonucléico Mensageiro
NCBI - *Nactional Center of Biotechnology Information (USA)*
NFκB - Fator Nuclear kappa B p50/p65
NO - Óxido Nítrico
$ONOO^-$ - Peroxinitrito
O_2 - Oxigênio molecular
O_2^- - superóxido
$PaCO_2$ - pressão parcial de dióxido de carbono
PAF - Fatores de Agregação Plaquetária
PCR - *Polymerase Chain Reaction*; Reação da Polimerase em Cadeia
RL - Radicais Livres
SIRS - Síndrome da Resposta Inflamatória Sistêmica
SOD - superóxido dismutase
SOD2 - Superóxido Dismutase dependente de Manganês
SOFA - *Sequential Organ Failure Assessment*
SNPs - *Single Nucleotide Polymorphism;* Polimorfismo em um único nucleotídeo
TCLE - Termo de Consentimento Livre Esclarecido
TLRs - *Toll-like receptors*
TNF-α - Fator de Necrose Tumoral Alfa
UTI - Unidade de Terapia Intensiva
UTIG HSL PUCRS - Unidade de Terapia Intensiva Geral do Hospital São Lucas da Pontifícia Universidade Católica do Rio Grande do Sul
Val - Valina

SUMÁRIO

CAPÍTULO 1 – INTRODUÇÃO E OBJETIVOS

1.1 INTRODUÇÃO

1.1.1 – Paciente crítico internado em UTI

Os pacientes internados na 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 que varia de 30% a 50% em sepse grave, e acima de 70% em choque séptico [Sands, *et al.,* 1997; Silva, *et al.*, 2004]. Em um estudo relativamente recente, estimou-se que nos Estados Unidos 50.000 pessoas morrem a cada ano decorrente de doenças críticas manifestadas nas UTIs como, por exemplo, a sepse, com custo total anual de até dez bilhões de dólares [Guha e Mackman, 2001]. A despeito dos progressos no diagnóstico e no tratamento das doenças infecciosas, a incidência de sepse tem aumentado nas últimas décadas. 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 [Alberti, *et al.,* 2002; Sands, *et al.,* 1997]. Apesar dos inúmeros progressos obtidos nas últimas décadas na tentativa de se dar suporte ao paciente crítico com foco infeccioso e sepse, a mortalidade neste grupo tem se mantido na faixa de 50% [Friedman, *et al.*,1998].

Sendo a sepse uma condição freqüente no âmbito da terapia intensiva, que culmina com elevada mortalidade e com tratamento com custo econômico elevado, sua abordagem é de interesse direto do sistema de saúde. O estudo da sepse deve, no entanto, contribuir para os levantamentos epidemiológicos e pautar-se numa abordagem direcionada para o conhecimento dos mecanismos moleculares e celulares que desencadeiam as variações fisiopatológicas. Este conhecimento básico poderá conduzir para a modulação da seqüência de eventos que culmina nos desfechos desfavoráveis como choque séptico e óbito. Conhecer as bases genéticas de tais eventos é, portanto, fundamental.

1.1.2 – Medicina Genômica

Há cerca de 20 anos, vários instrumentos de medida de predição de risco têm sido aplicados aos pacientes críticos internados em UTIs na tentativa de reconhecer as melhores estratégias terapêuticas. A avaliação do quadro crítico, nos dias de hoje, é principalmente realizada através de instrumentos que analisam a disfunção de órgãos e sistemas através do monitoramento diário de seu estado fisiológico. Um exemplo é o escore SOFA (*Sequentical Organ Failure Assessment*), o qual avalia diariamente a condição de seis sistemas orgânicos (respiratório, renal, hepático, hematopoiético, cardiovascular e neurológico), independentemente da terapia a qual o paciente está sendo submetido [Vincent, *et al.,* 1998]. No entanto, os citados instrumentos não consideram às predisposições genéticas de cada paciente.

Uma recente área das ciências da saúde é a chamada Medicina Genômica, cujo objetivo é identificar padrões genéticos que levam o indivíduo a ser mais ou menos suscetível a desenvolver alguma doença ou característica [Guttmacher e Collins, 2002]. Nos cerca de 20 mil genes do genoma humano [Wright *et al.*, 2001], codificadores de mais de 100 mil proteínas, já foram detectadas centenas de milhares de alterações (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, ou ainda, no caso de sepse, seu desfecho.

1.1.3 – Conceitos fundamentais para a definição do quadro séptico

A compreensão da fisiologia 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].

O episódio séptico leva em conta inúmeros fatores, mas sempre tem início em 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, de um monitoramento adequado e de um posterior tratamento para os pacientes. Assim, para sepse e condições relacionadas a sepse, consideram-se as definições a seguir (de acordo com os critérios propostos pela American College of Chest Physicians / Society of Critical Care Consensus Conference) [Boné, *et al*., 1992]:

- **Infecção** é definida como processo patológico causado por uma invasão de microorganismos patogênicos ou potencialmente patogênicos em tecidos estéreis, fluidos ou cavidades corporais;
- **SIRS** (Síndrome da Resposta Inflamatória Sistêmica) é diagnosticada como uma combinação de sinais clínicos apresentando, pelo menos, dois dos critérios a seguir: (I) Temperatura corporal >38ºC, ou temperatura corporal <36ºC; (II) Freqüência cardíaca >90 bpm; (III) Freqüência respiratória >20 irpm ou $PaCO_2$ <32 mmHg; (IV) Leucocitose, leucopenia, 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.

1.1.4 – Mecanismo celular na sepse

O sistema imunológico é muito complexo. O entendimento de como ele reconhece o próprio, o não próprio, inibe a resposta auto-imune e ao mesmo tempo

permite a reação do hospedeiro contra os invasores é o ponto chave da imunologia [Levy, *et al.,* 2003; López-Bojórquez, *et al.,* 2004].

A sepse inicia por uma resposta inflamatória que diretamente 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 aos receptores de reconhecimento padrão (*Toll-like receptors* TLRs) presentes na superfície das células do sistema imune humano. Após as ligações com TLRs, há a ativação de uma transdução de sinais que conduzem à ativação, no citoplasma, do fator nuclear de transcrição NFκB (*Nuclear Factor* κB), o qual é dirigido para o núcleo, onde ajuda a promover a transcrição de citoquinas como TNF-α (fator de necrose tumoral alfa), IL (interleucina)-1β, IL-6, IL-8, IL-12 e IFN- γ (interferon γ) [Russell, 2006].

Além das citoquinas, também ocorre liberação de óxido nítrico (NO), o qual desencadeia hipotensão (relaxamento endotelial) e ativação de fatores de agregação plaquetária (PAF) [Hoesel e Ward*,* 2004]. A atividade destes compostos pode conduzir à coagulopatia, o que dificulta a perfusão tecidual [Gomez-Jimenez, *et al.,* 1995], causa a queda significativa da pressão arterial e a 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]. Estas alterações hemodinâmicas combinadas induzem uma importante queda do transporte e da oferta de oxigênio aos tecidos, caracterizando o que se conhece por hipóxia estagnante.

Mesmo que a disponibilidade de oxigênio não estivesse comprometida, existem evidências na literatura que apontam para a disfunção mitocondrial como um dos principais mecanismos fisiopatológicos da disfunção celular e, conseqüentemente, da disfunção orgânica [Fink e Evans*,* 2002]. A produção aumentada de NO na célula durante o quadro séptico [Vincent, *et al*., 2000], bem como dentro da mitocôndria, traz como conseqüência a produção de radicais livres (RL) como o peroxinitrito ($ONOO^-$) [Boveris, *et al*., 2002], o qual provoca danos à Cadeia Transportadora de Elétrons [Pearce, *et al*., 2001]. Tais danos produzirão um

número aumentado de superóxido (O_2^-) [Liu, *et al.,* 2002; Taylor, *et al*., 1995; Guidot, *et al*., 1993]. O resultado final será a disfunção mitocondrial [Brealey, *et al*., 2002]. Enzimas antioxidantes como a superóxido dismutase (SOD) protegem a célula do dano oxidativo, tendo um grande potencial terapêutico para pacientes críticos [Salvemini e Cuzzocrea, 2003], porém, durante o quadro séptico, este balanço está desequilibrado, e o dano mitocondrial é inevitável [Gellerich, *et* al., 2002; Brealey e Singer, 2003]. A atividade da superóxido dismutase dependente de manganês (MnSOD), neste momento, é fundamental para a manutenção das funções mitocondriais [Williams, *et al.*, 1998].

1.1.5 – Superóxido Dismutase (SOD) (EC 1.15.1.1)

Espécies Reativas de Oxigênio (EROs) são moléculas altamente reativas, isso se deve às características do átomo e, consequentemente, à molécula de oxigênio que pode receber um elétron e se transformar no RL, denominada ânion superóxido (O_2^-), o qual pode gerar outras espécies reativas. Radicais livres e outras EROs são produzidos durante o metabolismo celular normal sendo potencialmente prejudiciais por possuírem a habilidade para reagir e alterar todos os componentes principais da célula, como lipídios, proteínas, carboidratos e ácidos nucléicos. A prevenção celular ao dano oxidativo é feita por enzimas antioxidantes (EAOs) como a SOD, a catalase e a glutationa peroxidase (GPx). O desequilíbrio entre oxidantes e antioxidantes é chamado de estresse oxidativo e tem um papel importante dentro da patogenicidade de muitas doenças críticas [Forsberg, *et al*.,2001].

A geração de EROs está bem relacionada à etiologia de uma diversidade de doenças, incluindo câncer [Jungst, *et al.*, 2004], aterosclerose [Harrison, *et al.*, 2003], doenças neurodegenerativas [Sheehan, *et al.*, 1997], lesão de isquemia-reperfusão [Zweier, *et al*., 1987], diabetes [Zotova, *et al*., 2003], entre outras, além de estar envolvida no processo de envelhecimento [Barja, 2004]. Está bem estabelecido que as EROs também possuem funções benéficas e são utilizadas pelas células como agentes de sinalização [Burdon, 1995; Thannickal e Fanburg, 2000]. No processo inflamatório, a produção de EROs tem seu lado positivo quando usadas como microbicidas pelas células fagocíticas [Diplock, *et al*., 1998], mas também seu

lado negativo, pois outras vias de produção de EROs são ativadas causando dano à célula [Seo, *et al*., 1995].

A compreensão dos mecanismos de ajuste fino entre produção e eliminação de EROs são objetivos de muitas pesquisas, nas quais a SOD é muito visada por combater o O_2^- , que é a primeira ERO a ser formada em condições fisiológicas normais e/ou patológicas [Boveris *et al*., 1972 apud Pitkänen e Robinson, 1998].

SOD é uma família de metaloproteínas onipresentes que catalisam a reação de dois ânions de superóxido (O_2^-) com a formação de peróxido de hidrogênio (H_2O_2) e oxigênio molecular (O_2) [Nordberg e Arnér, 2001]. Três tipos distintos de SODs foram identificados em células humanas [Zelko, *et al*., 2002]: 1) proteína homodimérica citosólica CuZnSOD [Crapo, *et al*., 1992], 2) proteína homotetramérica localizada na matriz mitocondrial MnSOD [Wan, *et al*., 1994], e 3) proteína homotetramérica extracelular EC-SOD [Folz e Crapo, 1994].

1.1.5.1 – Superóxido Dismutase Dependente de Manganês (MnSOD)

A enzima antioxidante MnSOD é codificada por um gene nuclear e 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]. Após ser sintetizada no citosol, a MnSOD é postranscricionalmente modificada e 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 sugerem que uma regulação intrínseca da MnSOD age sobre as outras enzimas antioxidantes [Van Remmen, *et al*., 1999].

1.1.5.2 – O gene humano SOD2

O gene humano que codifica a MnSOD, denominado SOD2, está localizado no braço longo do cromossomo 6 (6q25) [Church, *et* al., 1992] (Figura 1), possuindo cinco éxons e quatro íntrons [Wan, *et al*., 1994]. Foram localizados seis sítios

polimórficos [St. Clair, 2004]: 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].

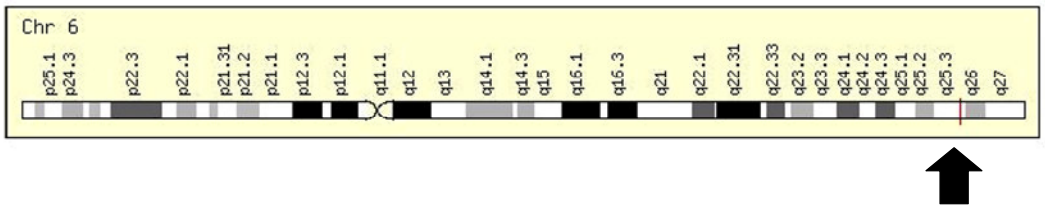

Figura 1: Posição do gene SOD2 no Cromossomo 6, indicado pela seta. Extraído do site ***GeneCard*** (for protein-coding SOD2: GC06M160020) http://www.genecards.org/ cgi-bin/carddisp.pl?gene=SOD2&search=obesity+OR+diabetes.

1.1.5.3 – Polimorfismo Ala-9Val (47C>T no gene SOD2)

Trata-se de um polimorfismo de um único nucleotídeo (*Single Nucleotide Polymorphism* SNP), ou seja, a substituição de uma base citosina (C) por uma base timina (T), no 47º nucleotídeo da seqüência de DNA que dará origem ao RNA mensageiro maduro. Esta mudança de nucleotídeo (47C>T) modifica o códon 16 de forma que haverá a incorporação de uma alanina (Ala; códon GCT) ou de uma valina (Val; códon GTT) no resíduo 16, pertencente à região do peptídeo sinal, ou na posição -9 a partir da contagem inversa do primeiro aminoácido da proteína madura [Rosenblum *et al.*, 1996]. Este polimorfismo gera uma alteração na conformação estrutural da enzima MnSOD: o alelo que codifica para alanina (Ala) adicionada na posição -9 da proteína madura gera uma estrutura conformacional de α-hélice, já o alelo que codifica para valina (Val) gera uma estrutura conformacional β-folha, a qual compromete a eficiência no transporte da MnSOD para a mitocôndria (ou seja, -9Ala pode ser mais facilmente transportado para a mitocôndria quando comparado com -9Val) [Shimoda-Matsubayashi, *et al.*, 1996; Shimoda-Matsubayashi, *et al.*, 1997]. 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].

Dentre os polimorfismos do gene SOD2, o Ala-9Val é o mais bem descrito na literatura e o que melhor reporta associações com diversas patologias.

A tabela 1 apresenta estudos buscando associação do polimorfismo Ala-9Val com diversas doenças e tipos de câncer.

Tabela 1: Relação de estudos com o polimorfismo 47C>T (Ala-9Val) SOD2 em populações Norte Americanas e Européias (A) e Asiáticas (B).

Autor, Ano (A)	**n**	**alelo Ala**	**Local**	**Doença**	**Associação**
Grasbon-Frodl *et al,* 1999	44 / 42	0.56	Germany	Parkinson	ns
Brans *et al,* 2005	157 / 201	0.50	Germany	ACD PPD	ns
Stickel *et al,* 2005	442 / 160	0.51	Germany/Austria	Cirrhosis	ns
Forunato *et al,* 2004	61 / 29	0.49	Italy	NIHL	ns
Martin *et al,* 2005	274 / 361	0.45	Poland	Gastric Câncer	ns
Ambrosone *et al,* 1999	266 / 295	0.55	USA	Breast câncer	Ala
Stoehlmacher *et al,* 2002	64 / 63	0.61	USA	Colorectal câncer	Ala
Millikan *et al,* 2004	2025 / 1812	0.48	USA	Breast câncer	Ala
Egan *et al,* 2003	470 / 497	0.51	USA	Breast câncer	ns
Levine *et al,* 2002	456 / 495	0.47	USA	Colorectal adenoma	ns
Liu *et al,* 2004	830 / 1119	0.50	USA	Lung câncer	Val
Tamimi *et al,* 2004	968 / 1205	0.50	USA	Breast câncer	ns
Farin *et al,* 2001	155 / 231	0.48	USA	Parkinson	ns
Wang *et al,* 2001	1101 / 1239	0.49	USA	Lung câncer	Val
Knight *et al,* 2004	643 / 612	0.49	Canada	Breast câncer	ns
Mitrunen *et al,* 2001	479 / 482	0.46	Finland	Breast câncer	Ala
Kakko *et al,* 2003	504 / 485	0.48	Finland	Atherosclerosis	Val
Woodson *et al,* 2003	197 / 190	0.51	Finland	Prostate câncer	Ala
Hirvonen *et al,* 2002	61 / 63	0.48	Finland	Pulmonary disorders	ns
Koistinen *et al,* 2006	89	0.50	Finland	AML	ns
Kinnula *et al,* 2004	248 / 193	0.52	Finland	Asthma	ns
Young *et al,* 2006	230 / 210	0.51	New Zealand	COPD	ns
Green *et al,* 2002	39 / 36	0.46	UK	Breast câncer	ns
Mattey *et al,* 2000	153 / 218	0.48	UK	Rheumatic arthritis	ns
Stewart *et al,* 2002	499 / 244	0.48	UK	AIOS, Liver fibrosis	ns
Holla *et al,* 2006	299 / 327	0.50	Czech Republic	Asthma	ns
Chistyakov *et al,* 2001	166 / 88	0.68	Russia	Diabetic neuropathy	Val
Zotova *et al,* 2003	86 / 94	0.58	Russia	Diabetic polyneuropathy	Val
Strokov *et al,* 2003	54 / 54	0.87	Russia	Diabetic polyneuropathy	Val
Masry *et al,* 2005	150 / 40	0.63	Egypt	Diabetic neuropathy	Val
Gurel *et al,* 2004	56 / 105	0.40	Turkey	Asthma	ns
Akyol *et al,* 2005	153 / 196	0.44	Turkey	Schizophrenia	Ala
Bergman *et al,* 2005	118 / 174	0.46	Sweden	Breast câncer	Val
Van Landeghem *et al,* 1999a	72 / 136	0.45	Sweden	Neuron motor diseases	Ala

Autor, Ano (B)	n	alelo Ala	Local	Doença	Associação
Hiroi *et al,* 1999	86 / 380	0.13	Japan	Non familial IDC	Val
Namikawa *et al,* 2004	63 / 150	0.15	Japan	NAS	Val
Hori *et al,* 2002	192 / 141	0.11	Japan	TD in schizophrenics	Ala
Shimoda-Matsubayashi *et al,* 1996	166 / 280	0.15	Japan	Parkinson	Ala
Kimura *et al,* 2000	99 / 197	0.19	Japan	Macular degeneration	Ala
Nomiyama *et al,* 2002	478 / 261	0.13	Japan	Diabetic neuropathy	Val
Cai *et al,* 2004	1125 / 1197	0.14	China	Breast câncer	Ala
Zhang *et al,* 2002	101 / 50	0.17	China	TD	ns
Mak *et al,* 2006	251 / 316	0.13	China	Asthma	ns
Van Landeghem *et al,* 1999b	476	0.30	China	Ethnics features	Ala [a]
Kim *et al,* 2005	106 / 115	0.15	Korea	Preeclampsia	ns
Pae *et al,* 2006	141 / 106	0.22	Korea	Mood disorders	ns
Hong *et at,* 2002	81	0.12	Korea	Oxidative damage	Ala
Yen *et al,* 2003a	70 / 93	0.20	Taiwan	Ankylosing spondylitis	ns
Yen *et al,* 2003b	43 / 92	0.17	Taiwan	Reactive arthritis	Val
Lin *et al,* 2003	198 / 332	0.16	Taiwan	Lung câncer	ns
Yen *et al,* 2003c	112 / 96	0.21	Taiwan	Rheumatoid arthritis	ns
Yen *et al,* 2003d	90 / 94	0.17	Taiwan	Lupus erythematosus	ns

n: número de casos / controles; ns: não significante; USA: United States of America; UK: United Kingdom; AIOS: Alcohol Induced Oxidative Stress; IDC: Idiopathic Dilated Cardiomyopathy; NAS: Non Alcoholic Steatohepatitis; TD: Tardive dyskinesia; ACD PPD: Allergic contact dermatitis by *para*-phenylene diamine; NIHL: Noise-Induced Hearing Loss; AML: acute myeloid leukemia; COPD: Chronic obstructive pulmonary disease; [a] Freqüência do alelo Ala em uma população Chinesa foi menor do que na população Européia. Fonte: O autor (2006).

Embora as EROs não sejam o único fator causal das patologias apresentadas, eles são componentes importantes de manifestações clínicas das doenças listadas. Estes resultados foram obtidos em diferentes etnias, sugerindo que certos *backgrouds* étnicos e diferentes tecidos podem ter influência sob a ação de cada um dos alelos em patologias específicas.

1.2 JUSTIFICATIVA

O desfecho favorável de um paciente criticamente doente e séptico será determinado por diferentes fatores como sua idade, suas condições clínicas, fisiológicas, celulares ou bioquímicas, unidas ao sucesso das intervenções terapêuticas. Numa mesma Unidade de Tratamento Intensivo, pacientes em condições de saúde similares podem vir a ter desfechos diferentes dependendo das respostas celulares de seu organismo. Assim, nesse momento, a herança genética pode representar um papel muito importante.

Enzimas antioxidantes como a Superóxido Dismutase dependente de manganês (MnSOD, codificada pelo gene SOD2) atuam protegendo a célula do dano oxidativo, condição celular típica durante a sepse. A atividade da MnSOD, em especial, pode determinar a funcionalidade da mitocôndria, o que é decisivo na patogêneses da sepse.

Dado que o gene SOD2 possui variantes gênicas que afetam a atividade mitocondrial da MnSOD, foi proposto que a herança diferencial poderia ter uma influência aditiva no momento da sepse e do estado crítico de saúde. Por esse motivo, elaborou-se essa pesquisa, a fim de determinar se seria possível medir tal influência, mesmo sendo a sepse um estado condicionado por múltiplos fatores.

1.3 OBJETIVOS

O objetivo do presente trabalho foi estudar o SNP 47C>T (Ala-9Val) do gene SOD2 que codifica para a superóxido dismutase dependente de manganês (MnSOD) em pacientes em condições críticas de saúde para: (I) buscar relações com ocorrência de sepse; (II) buscar relações com o desfecho a partir de sepse (choque séptico e mortalidade).

CAPÍTULO 2 – ARTIGO CIENTÍFICO

Title of the manuscript:

47C allele carriers (47C>T SNP; Ala-9Val mutation) of the SOD2 Gene were associated with Septic Shock

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Abstract:

In order to analyze the effect of the two different versions of the manganese superoxide dismutase gene (SOD2) on sepsis, we determined the 47C>T single nucleotide polymorphism (SNP) frequencies in critically ill patients with (n=356) and without sepsis (n=173), and also investigated the genotype frequencies in adverse outcomes in the septic patient's subgroup. We compared the 47C allele carriers (47CC+47CT genotypes) with 47TT homozygotes and we demonstrated a significant association between 47C allele carriers and septic shock in septic patients (p=0.025). With an adjusted binary logistic regression, incorporating 47C>T SNP and the main clinical predictors, we showed that only SOFA score [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. Our results and our hypothesis suggest that the higher 47C allele carrier frequency in septic patients with negative outcome is possibly explained by an effect on cellular stress.

Key words:

Critically ill patients; ICU; sepsis; septic shock; 47C>T SOD2 SNP; SOD2 Ala-9Val polymorphism.

INTRODUCTION

Into the cells, the mitochondria are an abundant source of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$) that is generated by incomplete reduction of molecular O_2. The $O_2^{\cdot-}$ is converted into a less reactive species, the hydrogen peroxide (H_2O_2), by the manganese superoxide dismutase (MnSOD, EC 1.15.1.1, codified by SOD2 gene) that is located in the mitochondrial matrix [1]. This antioxidant protection system is essential to mitochondrial function and cell arrangement: effective MnSOD activity is fundamental for the maintenance of mitochondrial stability. In addition, direct evidences correlate reduced MnSOD activity to the inadequate antioxidant defense and mitochondrial dysfunction [2].

During sepsis, both MnSOD and H_2O_2 are increased [3, 4, 5] since SOD2 expression is induced by oxidative stress, hypoxia, tumor necrosis factor (TNF-α), interleukin (IL)-1, and lipopolysaccharide (LPS) [6]. The excess of H_2O_2 is rapidly metabolized to O_2 and H_2O by glutathione peroxidase and catalase, but higher H_2O_2 concentration induces mitochondrial DNA single strand breaks, mitochondrial dysfunction, and the H_2O_2 oxidative cell damage occurs [7, 8].

Different effective MnSOD versions could affect the sepsis scenario if it influences the antioxidant defense system. The SOD2 human gene (*locus* 6q25) presents the 47C>T single nucleotide polymorphism (SNP) whose effect 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; refSNP ID: rs4880) [9]. The presence of alanine (-9Ala; 47C allele) is predicted to lead to higher mitochondrial MnSOD activity than the valine (-9Val; 47T allele) form [10]. 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) may lose the alpha-helical structure by a substitution at this residue [11, 12]. From this prediction, the -9Val MnSOD was suggested to be less efficiently transported into mitochondria than the -9Ala MnSOD [11], and that dimorphism

besides controlling the import also regulates the stability of the mRNA of MnSOD [13]. Hiroi et al [10] examined the mitochondrial processing efficiency directed by the -9Val MnSOD and -9Ala MnSOD leader signals of MnSOD and demonstrated that the -9Val MnSOD was, in fact, significantly less efficiently processed than the -9Ala MnSOD. Some population studies have suggested an important role of the 47C>T SOD2 SNP in human disease, but there is still inconsistency; disease associations in one population have not been confirmed in others (for example cancer in three North American populations) [14, 15, 16].

Recently, Elsakka et al [17] studying a sample of 40 septic patients found a reduced frequency of 47T allele (-9Val MnSOD) in patients with sepsis compared to healthy controls (n=100). Despite predictions from structural MnSOD protein studies, their pilot findings concluded that the 47C>T biallelic SNP in the SOD2 gene (Ala-9Val MnSOD polymorphism) had a functional effect. The authors argued that an inefficient targeting of MnSOD (the 47T allele trait) could result in the mitochondrial dysfunction observed in sepsis by inadequate $O_2^{\cdot -}$ conversion. However, on the other hand, it is also possible to propose that a more efficient MnSOD activity (the 47C allele trait) result in mitochondrial dysfunction too, due to excess H_2O_2 production. To analyze the efect of the two different SOD2 versions on sepsis, we determined the 47C>T SNP frequencies in patients with and without sepsis, and also investigated the genotype frequencies in adverse outcomes (septic shock and mortality) from sepsis in the septic patient's subgroup.

MATERIALS AND METHODS

Design and approval

This observational, hospital-based cohort study was conduced with data from 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, which receive about 300-400 patients/year. We work on the archived DNA collection from septic and non-septic ICU patients (controls). We

monitored patients daily during their entire ICU and post-ICU (hospital) stay. Patients were not eligible if they were diagnosed with HIV-infection, with know immunodeficiency, taking immunosuppressive drugs, pregnant, or lactating. This sepsis-genotyping project 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 patients or their surrogates.

DNA analysis

Genomic DNA was extracted from leucocytes by a standard method [18]. Genotyping protocols for the determination of 47C>T SOD2 SNP was previously described by [19]. Polymerase Chain Reaction (PCR) was performed at a total volume of 25 µL with about 10-100ng of genomic DNA, 1.6 U Taq DNA Polymerase in Taq Buffer (Life Technologies do Brasil Ltda. INVITROGEN Inv. São Paulo, SP, Brasil), final concentration of each dNTP 0.2mM, and 2mM $MgCl_2$, 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 do Brazil Ltda. INVITROGEN Inv. São Paulo, SP, Brasil) witch the underlined nucleotide represents the deliberate primer mismatches designed to introduce artificial restriction site [19]. The PCR was performed on an PTC-100 thermocycler (MJ Research, Inc. Watertown, MA, USA), as follow: an initial denaturation at 95ºC for 6 minutes, followed by 35 cycles at 95ºC for 1 minute, at 60ºC for 1 minute, and at 72ºC for 1 minute and 30 second. The final extension step was prolonged to 7 minutes. The 110bp PCR amplified product (25µL) was cleaved in appropriated buffer with 10U of the *Hae*III (GibcoBRL®-Life Technologies™, Rockville, MD, USA) at a total volume of 15µL at 37ºC for 8 hours. At least in 15% of samples were subjected to a second, independent PCR restriction fragment length-polymorphism analysis in order to confirm their genotypes.

We used in our lab routine 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 110bp 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.

Data collection

The patients were diagnosed for sepsis and sepsis-related conditions (severe sepsis and septic shock) according to the American College of Chest Physicians / Society of Critical Care Consensus Conference definition [20]. Sepsis was defined systemic inflammation, caused by infection, or occurring in the presence of clinical evidence of infection: septic patients were diagnosed with, at least, one infection focus, or had clinical evidence of infection, and were treated with wide spectrum antibiotics. Systemic inflammation (SIRS or the systemic inflammatory response syndrome) was defined by the presence of at least two of the following symptoms: Fever or hypotermia (temperature in the core oh the body >38°C or <36°C); Tachycardia (ventricular rate >90 heartbeats per minute); Tachypnea or Hyperventilation (>20 breaths/min or $PaCO_2$ <32 mmHg); Leucocytosis or leucopenia. If these symptoms were complicated by organ dysfunction, the definition of severe sepsis was fulfilled. If persistent arterial hypotension was present, the term septic shock was applied.

For illness severity evaluation we used the APACHE-II (Acute Physiology And Chronic Health Evaluation II) score [21] obtained on ICU admission day and used as an estimate for severity of disease. For organ dysfunction evaluation we used the SOFA (Sequential Organ Failure Assessment) [22] 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 ICU plus post-ICU (hospital) stay. For those patients with multiple ICU admission during the study period, only data from the first entrance was considered. Mortality was measured in days until death. For those patients with multiple ICU admission during the study period, only data from the first entrance was considered.

Statistical analysis

Statistical calculations were carried out 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 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. For the inclusion of variables in the multivariate model of logistic regression, we adopted, as a criterion, a correlation between successful aging and each independent variable at a significance level (p value) lower than 0.25 [23]. And 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.

RESULTS

During the period of the present study, and considering data from the first patient entrance, the ICU-HSL had 1911 patients with the incidence of sepsis in 64.3% (1229/1911). In the septic patient group, 65.3% (802/1229) had septic shock. This high incidence of sepsis and septic shock was attributed to the nature of this Medical-Surgery ICU.

We obtained data from 529 patients monitored daily from the ICU admission day to a maximum up to 224 days. Table 1 illustrates a complete description of the ICU patients (n=529) grouped according to the clinical phenotype: ICU patients with (n=356; 67.3%) and without (n=173; 32.7%) sepsis. Of these septic patients, 99.7% (355/356) were septic before ICU entrance (only one developed sepsis while in the ICU). Demographic, clinical and genetic characteristics were stated: the two groups had significant differences in the 13 parameters. The general genotypic frequencies in ICU sample were 47CC=0.24 (128/529); 47CT=0.50 (266/529); 47TT=0.26 (135/529) and the allelic frequencies were 47C=0.49 (522/1058); 47T=0.51 (536/1058), they did not differ from the values expected by the Hardy-Weinberg model (p=0.991). The frequencies from sub-samples obtained from patients with or without sepsis show that there were 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=0.978; without sepsis: 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=0.863)]. Comparing patients with and without sepsis, in our 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).

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 were stated: the groups had significant differences in different parameters. When we compared the three genotype groups (47CC, 47CT, 47TT) separately,

we found a trend with septic shock (p=0.078) and an unadjusted statistic association with mortality (p=0.022), and when we analyzed the 47C allele carriers group (47CC+47CT genotypes) against 47TT homozygotes we noticed 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 with mortality too (59.1 *vs* 46.7; p=0.040; OR=0.61, 95%CI=0.36–1.01). In the allele analysis septic shock, but not mortality (p=0.533), showed a trend to association with 47C allele (p=0.086).

We performed binary logistic regression to an adjusted analysis, incorporating both 47C carriers and 47TT homozygotes and main clinical predictors such as age and organ dysfunction (SOFA scores) 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 score [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 (final) of the forward stepwise (Wald) method showed that only SOFA score [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 logistic regression analysis 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 the Kaplan–Meier procedure using the 47C>T genotype groups as a discriminating factor. Taking all patients together, we observed that patients carrying the 47C allele did not have a significant worse outcome (Log-rank statistic, p=0.9147) when compared with those 47TT homozygotes. The same analysis was conduced on patients with sepsis (n=356) and septic shock (n=252) and the mortality distribution patterns were different although not statistically significant (Log-rank statistic, p=0.1944 and p=0.3250, respectively).

DISCUSSION

Our ICU patients' study revealed a significant association between the 47C>T SOD2 SNP and adverse outcome from sepsis. We compared the 47C allele carriers (47CC+47CT genotypes) with 47TT homozygotes and we demonstrated a significant association between 47C allele carriers and septic shock in critically ill septic patients.

The 47C>T SNP has a functional effect on MnSOD activity [10], but its phenotypical influences on sepsis, septic shock, or mortality are still unknown. In this study we did not find any association between sepsis and 47C>T SOD2 genotypes (p=0.800). In contrast, a positive association between 47T allele carriers and sepsis was found in a pilot study; Elsakka et al compared 40 septic patients (with four 47TT homozygotes) with 100 healthy controls [17]. The conflicting results could be based on: 1- the sample sizes were different (septic=356/non-septic=173 *vs* septic= 40/non-septic=100); 2- the patients were recruited from populations with diverse genetic background (United Kingdom *vs* southern Brazil); and 3- there were diverse control groups (ICU patients without sepsis *vs* healthy non-ICU volunteers). We assumed that the environmental exposure has a crucial influence.

We believe that MnSOD activity might have more influence on septic shock than on sepsis, e.g., the MnSOD variants could affect the susceptibility to septic shock from sepsis more than to sepsis from critical illness. During the sepsis the superoxide synthesis is increased; in some conditions of biologic stress, such as ischemia or sepsis in humans, uncoupling of electron transport may occur and $O_2^{\cdot -}$ production most likely increases even when $O_2^{\cdot -}$ tension is normal or low [5]. Likewise, the MnSOD expression is improved too. Because mitochondria are the principal O_2-consuming and ROS-generating organelles of the cell, Suliman et al postulated that cell activation by lipopolysacaride (LPS), which stimulates cytokine and ROS production, would damage mitochondria by oxidation of mitochondrial DNA (mtDNA). In testing their hypothesis in the liver, intraperitoneal LPS injection was found to cause a significant decrease in mtDNA copy number. LPS depleted glutathione

(GSH) and increased mitochondrial lipid peroxidation in conjunction with increased MnSOD gene expression [24].

Although our study is merely associative and it does not allow drawing definitive conclusion about cellular mechanisms, we would like to speculate that a more efficient MnSOD activity (the 47C allele trait) could result in mitochondrial dysfunction due to excess H_2O_2 production, but this complete mechanism can not be supported by our present data. The intracellular H_2O_2 exposure causes injury to epithelial cell as demonstrated by mtDNA single strand break formation [7]. Because it was predicted that the -9Ala MnSOD has higher activity than -9Val MnSOD [10] and it was found a functional effect of this polymorphism [17], we believe that 47C carrier may have an excess of H_2O_2 production which could lead to mtDNA damage of endothelial cell, improving the sepsis condition to result in septic shock and mortality. However, if it is acceptable, we fail to detect a critical independent association between 47CC homozygotes and these adverse outcomes. Possibly, it is because the elevated organ dysfunction rates in critically ill patients and the information that the 47C allele has some functional effect is only noticeable when it is considered a dominant-like model to 47C allele to perform the analysis.

In addition, we acknowledged other limitations of our study. First, patients are from a single center. Second, no haplotyped-based SOD2 gene approach was performed, thus does not ruling out the relationship of other SOD2 gene genetic variation with sepsis outcome. Third, we did not estimate patients MnSOD levels. Finally, the 47C allele can be linked with other gene polymorphism that acts synergistically or independently.

The number of recognized polymorphism involved in outcome from inflammatory circumstances is growing daily. We recognized that a study turns out to be more precise and real when it investigates a combination of genetic markers rather than analyzing only a single target, like our work. The single-marker studies are more limited by the fact that the polymorphism of a particular gene may be influenced by possibly linked variants at other *locus* or *loci*, or just be a marker for some confounding variables. Despit of this, our study

was able to detect a significant effect of -9Ala MnSOD under septic shock, showing that this unique SNP study may be biological reasonable, i.e., there is a plausible effect of the gene product in outcome from inflammatory condition. In addition, we can believe our methods have strength since we used a quality control system to ensure genotyping accuracy (sequencing verification of the DNA amplified fragment, black controls, and repetitions) in our lab routine, the lab technicians were blinded to phenotypes, the clinical investigators were blinded to genotypes, and we work with precise and universal definitions of sepsis and septic shock, worldwide acepted scores to organ dysfunction, and days to mortality determinations.

Lastly, we propose that the further SNP-array investigations should include the 47C>T SOD2 SNP alone or in combination with other functionally relevant mutations. 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) genes could also help to refine the understanding about septic shock predisposition. We are currently investigating some of these possibilities.

CONCLUSIONS

In conclusion, we showed that the 47C>T (Ala-9Val) SOD2 SNP was associated with adverse outcome from sepsis; there was higher frequency of septic shock in 47C allele carriers. Our results and our hypothesis suggest that the higher 47C allele carrier frequency in septic patients with negative outcome is possibly explained by an effect on cellular stress.

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PUCRS 2007-2008, and 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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Table 1 - Demographic, clinical and genotypic 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^{X2}
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)	0.000^{ST}
Admission cause - Surgical [n (%)]	86 (16.3)	47 (13.2)	39 (22.5)	0.821^{ST}
APACHE II Score [mean (SD)]	19.6 (7.9)	21.0 (7.0)	16.5 (8.2)	0.291^{ST}
SOFA-1 [median (min/max); mean (SD)]	6 (0/18); 6.5 (3.5)	8 (0/18); 7.5 (3.3)	4 (0/17); 4.6 (3.2)	0.000^{MW}
SOFA-2 [median (min/max); mean (SD)]	6 (0/18); 6.5 (3.6)	7 (0/18); 7.4 (3.5)	4 (0/14); 4.6 (3.1)	0.000^{MW}
SOFA-3 [median (min/max); mean (SD)]	6 (0/18); 6.4 (3.8)	7 (0/18); 7.3 (3.7)	4 (0/13); 4.6 (3.0)	0.000^{MW}
SOFA-4 [median (min/max); mean (SD)]	6 (0/19); 6.3 (3.8)	6 (0/19); 7.1 (3.8)	4 (0/14); 4.5 (3.1)	0.000^{MW}
SOFA-5 [median (min/max); mean (SD)]	5 (0/20); 6.2 (3.9)	6 (0/20); 6.9 (4.1)	4 (0/14); 4.4 (2.9)	0.000^{MW}
SOFA-6 [median (min/max); mean (SD)]	5 (0/21); 5.9 (3.9)	6 (0/21); 6.7 (4.0)	3 (0/14); 4.1 (2.9)	0.000^{MW}
SOFA-7 [median (min/max); mean (SD)]	5 (0/24); 5.9 (3.8)	6 (0/24); 6.5 (4.0)	4 (0/12); 4.1 (2.8)	0.000^{MW}
SOFA-15 [median (min/max); mean (SD)]	5 (0/19); 5.7 (4.0)	6 (0/19); 6.3 (4.2)	3 (0/10); 3.9 (2.7)	0.000^{MW}
SOFA-29 [median (min/max); mean (SD)]	4 (0/16); 5.7 (3.8)	6 (0/16); 6.3 (4.0)	3 (0/08); 3.4 (1.8)	0.004^{MW}
ICU LOS [days; median (min/max)]	15 (0/125)	15 (0/118)	11 (1/125)	0.000^{MW}
ICU+H 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)	
47CT [n (%)]	266 (50.3)	176 (49.4)	90 (52.1)	0.800^{X2}
47TT [n (%)]	135 (25.5)	91 (25.6)	44 (25.4)	
47CC+47CT [n (%)]	394 (74.5)	265 (74.4)	129 (74.6)	$0.975^{X2\ (a)}$
47TT+47CT [n (%)]	401 (75.8)	267 (75.0)	134 (77.5)	$0.536^{X2\ (b)}$
With 47C allele [n (%)]	522 (49.0)	354 (50.0)	168 (48.0)	0.722^{X2}
With 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^{X2}

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; ICU+H: 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; X2: Pearson Chi-Square 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^{X2}
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^{X2}
Admission cause - Medical [n (%)]	221 (87.7)	88 (84.6)	0.000^{ST}
Admission cause - Surgical [n (%)]	31 (12.3)	16 (15.4)	0.624^{ST}
APACHE II Score [mean (SD)]	21.6 (7.0)	19.2 (7.7)	0.004^{ST}
SOFA-1 [median (min/max); mean (SD)]	8 (1/18); 8.4 (3.1)	4 (0/13); 5.1 (2.6)	0.000^{MW}
SOFA-2 [median (min/max); mean (SD)]	8 (0/18); 8.3 (3.4)	4.5(0/15);5.1(2.8)	0.000^{MW}
SOFA-3 [median (min/max); mean (SD)]	8 (0/18); 8.2 (3.6)	5 (0/15); 5.1 (3.0)	0.000^{MW}
SOFA-4 [median (min/max); mean (SD)]	7 (0/19); 7.8 (3.9)	5 (0/14); 5.1 (2.7)	0.000^{MW}
SOFA-5 [median (min/max); mean (SD)]	7 (0/20); 7.7 (4.2)	4 (0/18); 4.9 (2.9)	0.000^{MW}
SOFA-6 [median (min/max); mean (SD)]	7 (0/21); 7.4 (4.0)	4 (0/17); 4.7 (3.0)	0.000^{MW}
SOFA-7 [median (min/max); mean (SD)]	7 (0/24); 7.0 (4.1)	4 (0/18); 4.9 (3.4)	0.000^{MW}
SOFA-15 [median (min/max); mean (SD)]	6 (0/19); 6.7 (4.2)	4 (0/16); 5.1 (3.8)	0.019^{MW}
SOFA-29 [median (min/max); mean (SD)]	6 (0/16); 6.8 (4.2)	4 (0/09); 4.5 (2.3)	0.096^{MW}
ICU LOS [days; median (min/max)]	15 (0/118)	13 (2/107)	0.014^{MW}
ICU+H 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^{X2}
47TT [n (%)]	56 (22.2)	35 (33.7)	
47CC+47CT [n (%)]	196 (77.8)	69 (66.3)	$0.025^{X2\ (a)}$
47TT+47CT [n (%)]	187 (74.2)	80 (76.9)	$0.536^{X2\ (b)}$
With 47C allele [n (%)]	261 (51.8)	93 (44.7)	0.086^{X2}
With 47T allele [n (%)]	243 (48.2)	115 (55.3)	
Mortality [n (%)]	161 (64.1)	37 (35.9)	0.000^{X2}

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^{X2}
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^{X2}
Admission cause - Medical [n (%)]	175 (88.4)	133 (85.3)	0.927^{ST}
Admission cause - Surgical [n (%)]	23 (11.6)	23 (14.7)	0.135^{ST}
APACHE II Score [mean (SD)]	23.0 (6.8)	18.3 (7.1)	0.000^{ST}
SOFA-1 [median (min/max); mean (SD)]	8 (1/16); 8.2 (3.2)	7 (0/18); 6.5 (3.2)	0.000^{MW}
SOFA-2 [median (min/max); mean (SD)]	8 (0/18); 8.2 (3.4)	6 (0/16); 6.4 (3.4)	0.000^{MW}
SOFA-3 [median (min/max); mean (SD)]	8 (0/18); 8.1 (3.8)	6 (0/18); 6.3 (3.4)	0.000^{MW}
SOFA-4 [median (min/max); mean (SD)]	7 (0/19); 8.1 (3.9)	5 (0/17); 5.8 (3.3)	0.000^{MW}
SOFA-5 [median (min/max); mean (SD)]	7.5(0/20);8.1(4.2)	5 (0/18); 5.4 (3.4)	0.000^{MW}
SOFA-6 [median (min/max); mean (SD)]	7 (0/21); 7.8 (4.1)	5 (0/16); 5.2 (3.3)	0.000^{MW}
SOFA-7 [median (min/max); mean (SD)]	7 (0/24); 7.6 (4.1)	4 (0/15); 4.9 (3.4)	0.000^{MW}
SOFA-15 [median (min/max); mean (SD)]	7 (0/19); 7.7 (4.2)	3 (0/16); 4.2 (3.2)	0.000^{MW}
SOFA-29 [median (min/max); mean (SD)]	7 (2/16); 7.3 (4.1)	3 (0/11); 4.7 (3.4)	0.007^{MW}
ICU LOS [days; median (min/max)]	16 (0/107)	14 (2/82)	0.122^{MW}
ICU+H 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^{X2}
47CC [n (%)]	45 (22.7)	43 (27.6)	
47CT [n (%)]	111 (56.1)	65 (41.7)	0.022^{X2}
47TT [n (%)]	42 (21.2)	48 (30.8)	
47CC+47CT [n (%)]	156 (78.8)	108 (69.2)	$0.040^{X2\ (a)}$
47TT+47CT [n (%)]	153 (77.3)	113 (72.4)	$0.296^{X2\ (b)}$
With 47C allele [n (%)]	201 (50.8)	151 (48.4)	0.533^{X2}
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; ICU+H: 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; X2: Pearson Chi-Square 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 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 (n=356).

(A) All critically ill patients (n=529)				
Step	**Variable**	**Odds ratio* (95% CI)**	***p***	**Percent of correct prediction**
1	Category SOFA	10.171(6.655-15.545)	<0.001	74.2
2	Category SOFA	10.677(6.942-16.422)	<0.001	74.2
	47C allele	1.748 (1.108-2.758)	0.016	
(B) Septic patients (n=356)				
Step	**Variable**	**Odds ratio* (95% CI)**	***p***	**Percent of correct prediction**
1	Category SOFA	8.516 (5.042-14.383)	<0.001	77.7
2	Category SOFA	9.107 (5.319-15.592)	<0.001	77.7
	47C allele	2.125 (1.190-3.794)	0.011	

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

CAPÍTULO 3 – CONSIDERAÇÕES FINAIS

A sepse é uma síndrome complexa que sofre a interferência de múltiplos fatores. Centenas ou milhares de fatores externos e de fatores intrínsecos podem determinar simultaneamente a susceptibilidade e o desfecho de uma condição crítica como a sepse. Cada efeito externo e cada um dos genes herdados exercerão isoladamente um pequeno efeito, mas que cumulativamente, no somatório com os demais, definirá o desfecho. O estado de saúde, o prognóstico e o desfecho de pacientes com sepse estão, portanto, também relacionados à herança genética que o indivíduo recebeu.

Ainda que centenas de genes possam estar envolvidos na modulação fisiológica de um paciente crítico, aqueles genes que interferem sistemicamente são sempre muito decisivos. Nós reconhecemos que um estudo se revela mais preciso e real quando ele investiga uma combinação de marcadores genéticos ao invés de uma análise de um único alvo. Os estudos de marcadores únicos (SNP) são mais limitados pelo fato de que o polimorfismo de um gene particular pode ser influenciado através de variantes possivelmente unidas em outro(s) *locus* ou *loci*, ou realmente ser um marcador para algumas variáveis contraditórias. Mesmo assim, nosso estudo foi capaz de detectar um efeito significativo do alelo 47C sobre choque séptico, mostrando que o estudo deste único SNP possa ter plausibilidade biológica.

Como apresentado ao longo deste trabalho, nosso objetivo foi responder à pergunta se uma variante polimórfica do gene que codifica para a MnSOD poderia influenciar no desfecho de sepse em uma amostra de pacientes críticos residentes no Sul do Brasil. Verificou-se que o SNP 47C>T (Ala-9Val) SOD2 não estava associado a risco à sepse, mas estava associado ao tipo de desfecho a partir da sepse: houve uma freqüência mais alta de desfecho negativo (choque séptico e mortalidade) em portadores do alelo 47C. Nossos resultados e hipótese sugerem que a maior freqüência de desfechos negativos (choque séptico e mortalidade) em portadores do alelo 47C pode ser explicada por um efeito de estresse celular.

Finalmente, ainda não está claro qual o real mecanismo de sinalização que leva ao dano e, conseqüentemente, à morte celular gerada pelo estresse oxidativo que é produzido durante a sepse e o choque séptico. Baseados nas informações fornecidas pela literatura atualizada e nos achados do nosso grupo, especulamos uma hipótese, a qual foi apresentada nesse trabalho.

Porém outros estudos serão necessários para confirmar essa hipótese, afim de melhor compreender o possível efeito das variantes -9Ala e -9Val da MnSOD nas células durante o quadro de sepse.

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