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DISSERTAÇÃO DE MESTRADO:

Estudo das variantes polimórficas -786T>C e 894G>T (Glu298Asp) do gene que codifica para a sintase do óxido nítrico endotelial (eNOS) e a ocorrência de sepse, choque séptico e disfunções orgânicas em pacientes com condições críticas de saúde.

Trabalho apresentado com a finalidade de obtenção de Título de Mestre em Biologia Celular e Molecular pelo PPGBCM-PUCRS

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RESUMO

Objetivo: Investigar se há associação entre as variantes polimórficas -786T>C e 894G>T (Glu298Asp) do gene que codifica para a sintase do óxido nítrico endotelial (eNOS) e a ocorrência de sepse, choque séptico e disfunções orgânicas em pacientes com condições críticas de saúde. **Pacientes e Métodos:** Foram selecionados para esse estudo 207 pacientes críticos internados na unidade de tratamento intensivo geral (UTI) do Hospital São Lucas da PUCRS, admitidos de março de 2002 a dezembro de 2005. O grupo controle foi constituído por 149 doadores voluntários saudáveis oriundos da mesma população. A disfunção orgânica dos pacientes sépticos foi avaliada durante a primeira semana após admissão na UTI, através do escore SOFA, e foram consideradas as ocorrências de sepse, choque séptico e óbito. Os genótipos das variantes polimórficas -786C>T e 894G>T foram determinados por análise de fragmentos de restrição dos produtos da reação em cadeia da polimerase (PCR). Após, foi analisada a freqüência da distribuição dos genótipos e dos alelos entre os grupos de pacientes e de indivíduos saudáveis. **Resultados:** A freqüência de portadores do alelo -786C entre os pacientes críticos e pacientes sépticos foi significativamente mais alta do que entre os voluntários saudáveis (58% *vs* 46%; *P*=0,021, OR=1.64, $CI_{95\%}$: 1.05-2.57, e 60% *vs* 46%; *P*=0,012, OR=1.82, $CI_{95\%}$: 1.10-3.01, respectivamente). Homozigotos -786CC tiveram significativamente um maior grau de disfunção orgânica, medido pelo escore SOFA durante a crucial primeira semana de internação na UTI (*P*=0,001) sem, no entanto, haver diferença significativa na taxa de mortalidade. Homozigotos 894TT foram mais freqüentes entre os pacientes críticos e pacientes sépticos do que entre indivíduos saudáveis (25% *vs* 14%; *P*=0,017, OR=0.49, $CI_{95\%}$: 0.25-0.92, e 25% *vs* 14%; *P*=0,024, OR=0.46, $CI_{95\%}$: 0.22-0.96, respectivamente). Por outro lado, portadores do alelo 894G tiveram escores SOFA significativamente mais altos (*P*=0,028) e não houve relação significativa com a mortalidade. Detectou-se a presença significativamente superior do duplo-homozigoto -786CC/894TT no grupo de pacientes (*P*=0,002) se comparada ao grupo de sujeitos saudáveis, e o duplo-homozigoto 786TT/894GG significativamente mais freqüente no grupo de voluntários (*P*=0,002) do que no grupo de pacientes. **Conclusão:** Ambas variantes polimórficas -786C>T e 894G>T da eNOS podem estar associadas com um maior risco de suscetibilidade ao desenvolvimento de condições clínicas críticas mais graves, à sepse e ao choque séptico.

FUNDAMENTAÇÃO TEÓRICA

1 - Pacientes críticos internados em Unidade de Terapia Intensiva (UTI)

Os pacientes internados em Unidades de Terapia Intensiva (UTI) caracterizam-se 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% (Vincent *et al.,* 2002). Nos últimos 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. O escore SOFA (*Sequential Organ Failure Assessment*) avalia diariamente a condição de seis sistemas orgânicos (respiratório, renal, hepático, hematopoiético, cardiovascular e neurológico), independentemente da terapia à qual o paciente está sendo submetido (Vincent *et al.,* 1998).

Dado que pacientes de UTIs são indivíduos afetados por múltiplas disfunções orgânicas e que, além disso, estão expostos ao ambiente hospitalar o qual é rico em diversidade de microorganismos infecciosos, o risco de que esses pacientes venham a desenvolver uma infecção é muito elevado. Uma parcela expressiva de pacientes desenvolve infecção bacteriana sendo que cerca de 60% acaba por desencadear quadros de sepse, choque séptico, disfunção e falência de múltiplos órgãos. O quadro de sepse é conseqüência de processos celulares em resposta a uma agressão de origem infecciosa. A manifestação clínica da sepse pode agravar-se chegando a um quadro de choque, o qual caraceriza-se pela presença de

vasodilatação periférica acentuada e por excessiva presença de agentes pró-inflamatórios que, juntos, acentuam ainda mais a disfunção e a falência de múltiplos órgãos.

Detectam-se evidências de disfunção muito antes da falência de órgãos, resultado da reação inflamatória pela massiva liberação de citocinas. A resposta sistêmica à infecção é mediada através das citocinas derivadas de macrófagos que alvejam os receptores da extremidade-órgão em resposta a ferimento ou infecção (Bone *et al.,* 1997; Haddad *et al.,* 2002). Tal resposta inflamatória à infecção ou ao ferimento é uma reação altamente conservada e regulada do organismo. A liberação concomitante de agentes pró-inflamatórios e antiinflamatórios mantém a homeostasia do organismo. A reação antiinflamatória pode ser maior e algumas vezes mais longa que a pró-inflamatória; o objetivo disto é diminuir a síntese de agentes pró-inflamatórios, conservando o equilíbrio homeostático (Bone *et al.,* 1997; Haddad *et al.,* 2002).

Apesar dos inúmeros progressos obtidos nas últimas décadas na tentativa de se dar suporte ao paciente crítico com foco infeccioso e sepse, a mortalidade nesse grupo tem-se mantido na faixa de 50% (Friedman *et al.,* 1998).

Sendo a sepse uma condição freqüente no âmbito da terapia intensiva, que causa elevada taxa de mortalidade e tem tratamento com custo muito alto, sua abordagem é de interesse direto do sistema de saúde. O estudo da sepse deve contribuir para os levantamentos epidemiológicos e pautar-se por uma abordagem direcionada para o conhecimento dos mecanismos celulares e moleculares que desencadeiam as variações fisiopatológicas. Esse conhecimento básico poderá contribuir para a modulação da seqüência de eventos que culmina nos desfechos desfavoráveis. Nesse sentido, conhecer as bases genéticas de tais eventos é condição fundamental.

2 – Sepse e óxido nítrico

2.1 Sepse

A sepse caracteriza-se por uma resposta inflamatória sistêmica que é crucial para a disfunção múltipla de órgãos (Anderson, 2005). Tal resposta é causada após uma infecção bacteriana que ativa o sistema imunológico e, mesmo com os recentes avanços no tratamento intensivo e descoberta de novos antibióticos, ela ainda permanece associada a uma alta taxa de mortalidade (Anderson, 2005; Hubacek *et al.,* 2001).

Nos anos 90, a sigla SIRS (Síndrome Sistêmica da Resposta Inflamatória) foi definida para fazer referência à resposta imunológica sistêmica causada por episódios inflamatórios (Karima *et al.,* 1999). A sepse é considerada SIRS induzida por infecção e, se o quadro não for revertido, pode evoluir para choque séptico (hipotensão refratária) e óbito por disfunção sistêmica (MODS) seguida de falência múltipla de órgãos (MOF) (Karima *et al.,* 1999).

O controle do fluxo sangüíneo é feito pela interação de substâncias vasoconstritoras e vasodilatadoras (Berthold *et al.,* 1999). Durante a evolução do quadro séptico é crucial o monitoramento dos níveis de oxigênio nos pacientes, sendo o aporte desse gás, nos níveis da macro e da microcirculação, essencial para que não haja morte tecidual por necrose (Morisaki *et al.,* 2004).

O entendimento da regulação fisiológica, bem como dos fatores envolvidos no controle do tônus vascular, tanto genéticos quanto bioquímicos faz-se importante para a compreensão da fisiologia da sepse.

2.2 Óxido Nítrico

O óxido nítrico (NO) é um poderoso agente vasodilatador sintetizado a partir da oxidação da L-arginina pela ação da enzima sintase do óxido nítrico (NOS) (Afrasyap *et al.,* 2004). São atribuídas ao NO funções como inibição da ativação

plaquetária, indução do crescimento e proliferação das células da musculatura lisa, iindução de adesão leucocitária e oxidação das lipoproteínas de baixa densidade (LDLs) (Afrasyap *et al.,* 2004; Colombo *et al.,* 2002).

Existem três isoformas de enzimas denominadas Sintases do Óxido Nítrico (NOS): endotelial (eNOS), induzível (iNOS) e neuronal (nNOS) (Moncada *et al.,* 1991). As isoformas endotelial e neuronal são expressas constitutivamente e ativadas pelo aumento de íons cálcio no meio intracelular, estando envolvidas no controle do tônus vascular e na transmissão de sinais (Moncada *et al.,* 1991). A iNOS é expressa em resposta à liberação de citocinas pró-inflamatórias quando na presença de uma infecção. Acredita-se que ela seja responsável pela grande quantidade de NO liberado, o que pode levar à lesão tecidual e MODS (Fatini *et al.,* 2004; Knowles *et al.,* 1992; Gomez-Jimenez *et al.,* 1995).

A meia vida da molécula de NO é muito curta, convertendo-se assim, rapidamente, em nitrito (NO^-_2) e nitrato (NO^-_3), metabólitos que são utilizados como marcadores para os níveis plasmáticos de NO e que causam dano oxidativo por atuarem como radicais livres (Afrasyap *et al.,* 2004; Kim *et al.,* 2003; Levecque *et al.,* 2003).

Durante a endotoxemia foi evidenciada grande concentração de nitrato no plasma e na urina de pacientes com sepse. Constatou-se, também, que esses níveis aumentam quando o paciente desenvolve choque séptico. Isso evidencia uma relação clínica quantitativa e qualitativa entre a produção de NO, endotoxemia, disfunção hemodinâmica e MODS no choque séptico (Kilbourn *et al.,* 1993).

O aumento de NO está associado com peroxidação lipídica, causando alterações celulares e nitrosilação protéica, responsável pela alteração da funcionalidade das proteínas (Levecque *et al.,* 2003).

A relevância da superprodução de NO e o desenvolvimento de choque séptico têm sido demonstrada na utilização de inibidores da NOS e agentes que bloqueiam a expressão da iNOS como o L-NMMA (N^{ω}-metil-L-arginina) que promoveu a restauração da pressão sangüínea revertendo a hipotensão causada por endotoxinas devido ao aumento da resposta a vasoconstritores. Esses inibidores também melhoraram a função hepática e renal em modelos animais quando tratados com LPS, LTA e peptidoglicanos (Salkowski *et al.,* 1997).

Estudos de indução de sepse em ratos com LTA demonstram que a expressão do mRNA da eNOS aumenta consideravelmente (Rackow *et al.,* 1991).

O NO também é considerado um mensageiro biológico por estimular quinases dependetes de GMPc e aumentar a expressão da enzima guanilato ciclase (Noiri *et al.,* 2002; Novoradowsky *et al.,* 1999). Muitos estudos estão sendo realizados tentando elucidar a relação de polimorfismos genéticos e predisposição a doenças. Novas tecnologias vêm contribuindo para identificação dessas variantes polimórficas (Naber *et al.,* 2001), e sempre que se analisam as bases genéticas ou a origem molecular de uma desordem deve-se levar em conta o ambiente, a etnia e a genética individual.

Estima-se que mais de 400 genes estejam envolvidos em processos como função endotelial e inflamação (Doevedans PA, *et al.,* 2001). No que se refere ao tônus vascular, uma maior compreensão acerca do controle do mesmo é de fundamental importância em um quadro de infecção sistêmica e pode influenciar no desfecho do paciente, visto que uma vasodilatação em excesso pode conduzir ao choque.

Inibidores da eNOS como o L-NAME (N^{G}-nitro-L-arginina metil ester) induzem hipertensão e o aparecimento de lesões ateroscleróticas que podem ser revertidas

pela administração de L-arginina (Hingorami *et al.,* 1999; Hibi *et al.,* 1998; Benjafield *et al.,* 2000).

Mediadores inflamatórios como TNF-α, IL-1 e IFN-γ estimulam a produção de NO. Células tumorais tratadas *in vitro* com estes mediadores, tiveram induzida a sua produção de NO que induziu apoptose nas mesmas (Hingorami *et al.,* 1999). Considerando que durante o desenvolvimento da sepse, tais mediadores imunológicos são liberados pelo corpo em resposta a uma infecção (Anderson, 2005), pesquisas acerca do NO fazem-se importantes no sentido de buscar elucidar a sua relação, como um desses mediadores, durante o quadro de SIRS.

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 30 milhões de mortes anuais 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. Ele pode atingir indivíduos de qualquer idade ou população, mas sempre tem início por um processo infeccioso (geralmente bacteriano) que, por sua vez, desencadeia uma inflamação sistêmica denominada SIRS (Síndrome Sistêmica da Resposta Inflamatória) (López-Bojórquez *et al.,* 2004; Levy *et al.,* 2003; Friedman *et al.,* 1998; Karima *et al.,* 1999).

Considera-se infecção todo e qualquer processo patológico causado por uma invasão de patógenos em tecidos estéreis, fluidos ou cavidades corporais (Levy *et al.,* 2003). Essa inflamação pode provocar uma bacteremia, a qual consiste na presença de atividade bacteriana no sangue. O sistema imunológico reconhece os patógenos pela interação das moléculas extracelulares. Bactérias Gram-negativas

são reconhecidas pelo CD14 (*Cluster of Differentiation* 14), um receptor de monócitos, macrófagos e neutrófilos (Hoesel *et al.,* 2004), através de suas endotoxinas: as LPS (lipopolissacarídios). As bactérias Gram-positivas por suas moléculas de LTA (ácido lipoteicóico) e, em uma eventual infecção por fungos, os ergosteróis presentes em sua parede celular são moléculas alvo na ativação do sistema imunológico (López-Bojórquez *et al.,* 2004).

A partir desse quadro fisiopatológico, o paciente pode evoluir para sepse, sepse severa, SIRS, choque séptico e disfunção/falência de múltiplos órgãos (MODS/MOF) (López-Bojórquez *et al.,* 2004; Levy *et al.,* 2003; Friedman *et al.,* 1998).

A SIRS é diagnosticada pelos seguintes parâmetros clínicos: (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 céls/mm^3, ou presença de > 10% de formas jovens (bastões) (Akira 2000; Takeuchi *et al.,* 2001; Medzhitov *et al.,* 2001).

A síndrome da disfunção de múltiplos órgãos consiste na deterioração generalizada (falência metabólica) de órgãos, aumento do metabolismo basal, alteração na homeostase e decréscimo rápido da massa corporal) (López-Bojórquez *et al.,* 2004).

4 – A fisiopatologia da sepse

O sistema imunológico é muito complexo. O entendimento de como ele reconhece o próprio, o não próprio, inibe 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 partir do momento em que uma bactéria (ou qualquer outro patógeno) entra no organismo, monócitos e macrófagos são ativados e recrutados para o reconhecimento dos antígenos bacterianos (Karima *et al.,* 1999; Netea *et al.,* 2003; Fujihara *et al.,* 2003). Tal reconhecimento é feito pela interação das moléculas extracelulares bacterianas com receptores dessas células (CD14 e proteínas glicosiladas em macrófagos) (Karima *et al.,* 1999; Netea *et al.,* 2003; Fujihara *et al.,* 2003). Existe também um grupo de receptores, os *toll-like receptors* (TLR), que participam dessa etapa de reconhecimento dos antígenos (Karima *et al.,* 1999; Netea *et al.,* 2003; Fujihara *et al.,* 2003). Entre a variedade de TLRs, a descrição de alguns exemplos deles é facilmente encontrada na literatura: o TLR-4 que reconhece LPS e o TLR-2 que reconhece LTA e peptidoglicanos (Netea *et al.,* 2003). O LTA é componente da parede celular de bactérias gram-positivas (Wang *et al.,* 2000). Além deles, o TLR-9 reconhece e liga-se ao DNA bacteriano (Takeuchi *et al.,* 2001; Medzhitov *et al.,* 2001; Wang *et al.,* 2000; Netea *et al.,* 2003; Titheradge *et al.,* 1999; Moncada *et al.,* 1991). Após o reconhecimento dos patógenos, macrófagos e monócitos desencadeiam uma comunicação intracelular que ativa a síntese de fatores de transcrição como o fator nuclear de transcrição NFκB (*Nuclear Factor* κB) e o NF-IL-6 (*Nuclear Factor* interleucina–6) envolvidos na transcrição de inúmeras citocinas. Assim, são sintetizadas e secretadas citocinas como TNF-α (fator de necrose tumoral alfa), IL (interleucina)-1β, IL-6 e IL-8, IL-12 e IFN- γ (interferon γ). As citocinas são fatores solúveis que atingem outros tipos celulares promovendo

alterações internas em tais células além de ativarem linfócitos T e células CD4 que também secretam tais substâncias e participam no combate de células tumorais (Levy *et al.,* 2003; Akira 2000; Carreras *et al.,* 2004; López-Bojórquez *et al.,* 2004; Hoesel *et al.,* 2004; Knowles *et al.,* 1992; Gómez-Jimenez *et al.,* 1995). Consta também na literatura que o TNF-α e a IL-1 afetam diretamente a contractibilidade do miocárdio (Gómez-Jimenez *et al.,* 1995; Carreras *et al.,* 2004).

Além das citocinas, também ocorre liberação de óxido nítrico (NO) que causará hipotensão (relaxamento endotelial) e ativação de fatores de agregação plaquetária (PAF) (Hoesel LM, *et al.,* 2004). A atividade destes compostos pode conduzir à coagulopatia o que dificulta a perfusão tecidual determinando uma seqüência de eventos que podem levar a MODS (Figura 1) (Gomez-Jimenez J, *et al.,* 1995).

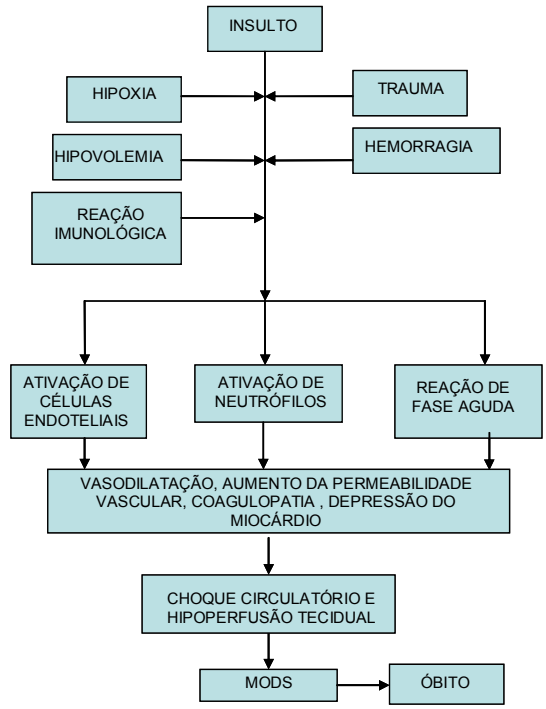

Figura 1: Seqüência de eventos inflamatórios passando pela SIRS, MODS e culminando com óbito (Gomez-Jimenez *et al.,* 1995).

5 - Os estágios do quadro séptico:

Muitas vezes podemos observar no quadro séptico o aparecimento de estágios progressivos e complexos causadores de desordens no sistema imunológico, desencadeados pela infecção. Existem alguns fatores que podem levar a SIRS: infecção, trauma, queimaduras, isquemia, reperfusão e inflamação. Tal síndrome é caracterizada por um desequilíbrio no sistema imunológico (hiperatividade ou hipoatividade) (Knowles *et al.,* 1992).

5.1 - O estágio inicial da sepse (fase hiperdinâmica).

Caracteriza-se pela ativação intensa de citocinas pró e antiinflamatórias. Essa fase é marcada pela produção de interleucinas (IL-1, IL-6, IL-8) e do fator de necrose tumoral TNF-α. Também há aumento nos níveis de proteína C-reativa que acredita-se ser responsável pela ativação das espécies reativas de oxigênio e pela produção de óxido nítrico (Hoesel *et al.,* 1994; Knowles *et al.,* 1992).

5.2 - O estágio tardio da sepse (fase hipodinâmica).

Essa etapa é marcada pela liberação das interleucinas IL-10 e IL-13 para conter o efeito pró-inflamatório da fase hiperdinâmica, suprimindo a ativação do NFκB. Há também um fenômeno conhecido como CARS que consiste em uma resposta inflamatória compensatória. No final desse estágio há diminuição da fagocitose dos macrófagos, quimiotaxia, produção de citocinas e de radicais livres (Baghat *et al.,* 1999).

6 – O gene da eNOS

O gene da eNOS está localizado no *locus* 7q35-36, contendo 26 exons totalizando 21kb onde já foram localizadas algumas variações polimórficas (Figura 2). Na região promotora encontram-se, pelo menos, três SNPs: -1468T>A, 922A>G e -786C>T. Foram detectadas, pelo menos, quatro mutações intrônicas: 18 27A>C no intron 1, repetições CA no intron 13, 10G>T no intron 23 e um VNTR (nucleotídeos variados repetidos em tandem) no intron 4. No exon 7 existe um SNP na posição 894G>T (Afrasyap *et al.,* 2004; Kim *et al.,* 2003; Bilsborough *et al.,* 2003; Salkowski *et al.,* 1997).

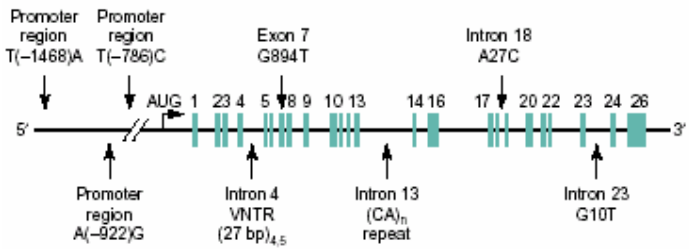

Figura 2: Polimorfismos existentes no gene da eNOS (Carreras *et al.,* 2004).

O polimorfismo do exon 7 (894G>T) resulta na modificação no resíduo 298 da proteína final que consiste numa substituição de um ácido glutâmico por um ácido aspártico (Glu298Asp) e seus genótipos estão associados a diferenças na capacidade de vasodilatação endotelial. O alelo 894G, por ter uma eNOS supostamente mais funcional, vem sendo associado a uma maior vasodilatação quando comparado ao alelo 894T, que possui uma relatada menor atividade da eNOS (Afrasyap *et al.,* 2004).

Em ratos *knock-out* para o gene de eNOS há uma maior incidência de doenças tromboembólicas e menor resposta à acetilcolina (Noiri *et al.,* 2002; Bucher

et al., 1997). A inibição ou deterioração da eNOS eleva o fluxo sanguíneo em humanos levando à hipertensão arterial (Salkowski CA, *et al.,* 1997). Em indivíduos com tumor prostático, pacientes 894GG, apresentaram um maior número de metástases disseminadas pelo organismo devido a uma maior vasodilatação que os indivíduos 894TT (Bilsborough *et al.,* 2003).

Estão associadas ao genótipo 894TT patologias como doença arterial coronariana (CAD), espasmo coronariano, aterosclerose e aterogênese, além de outras alterações patológicas pela razão de o alelo 894T ser menos funcional, quando comparado ao 894G (Colombo *et al.,* 2002; Hingorami *et al.,* 1999; Harkema *et al.,* 1990; Rackow *et al.,* 1991; Brown *et al.,* 1997).

Estuda-se, porém, se o SNP 894G>T é de fato o responsável pelas alterações fisiológicas da eNOS ou se ele representa apenas um marcador estando ligado a outra variante polimórfica que seria a real causadora da alteração na eNOS e, consequentemente, da susceptibilidade às patologias (Benjafield *et al.,* 2000; Bilsborough *et al.,* 2003; Poderoso *et al.,* 1996).

Além do polimorfismo do exon 7, existe um outro SNP que tem sido foco de muitas pesquisas no estudo da eNOS: o SNP -786C>T localizados na região promotora da eNOS. Foi sugerido que essa mutação reduz em até 50% da atividade promotora o que poderia influenciar de maneira direta na expressão do gene (Castro L, *et al.,* 1994; Sandrim VC, *et al.,* 2006). Assim o alelo -786C poderia ser responsável por uma diminuição nos níveis séricos de NO.

Alguns pesquisadores têm observado que essa diminuição não é significativa. Em asiáticos o SNP -786C>T está associado com espasmo coronariano e enfarte do miocárdio (Jo *et al.,* 2006; Yoon *et al.,* 2005). Por outro lado essa associação não se encontra em franceses e australianos (Granath *et al.,* 2001). Em um estudo sobre a

influência dos haplótipos da eNOS com hipertensão, verificou-se que novamente o alelo -786C seria a causa da atividade promotora deficiente (Sandrim *et al.*, 2006).

Em italianos foi comprovada a relação desses dois SNPs com a incidência e a severidade de CAD. De acordo com os pesquisadores, as pessoas que carregam o alelo -786C do polimorfismo –786C>T e o alelo 894T do polimorfismo 894G>T constituem uma população de alto risco para o desenvolvimento de CAD (Colombo *et al.*, 2003).

Acreditamos que a eNOS pode estar de alguma forma relacionada com o quadro clínico do paciente crítico devido ao seu papel fisiológico no controle do tônus vascular. A partir disso, o estudo de seus polimorfismos pode ser uma ferramenta clínica para o prognóstico destes pacientes e futuramente pode conduzir os médicos à realização de um tratamento mais específico baseado nas características genéticas individuais.

7 - Referências bibliográficas

1. Afrasyap L, Ozturk G. NO level and Endothelial NO Synthase Gene Polymorphism with Coronary Artery Disease from the Turkish Population. Acta Biochimica et Biophysica Sinica, 2004; 36: (10) 661-666.
2. Akira S. Toll like receptors: lessons from knockout mice. Bichem Soc Trans, 2000; 28(5):551-556.
3. Anderson J. The inflamatory reflex – Introduction. Journal of Internal Medicine, 2005; 257:122-125.
4. Benjafield V, Morris BJ. Association analyses of endothelial nitric oxide synthase gene polymorphisms in essential hypertension. American Journal of Hypertension, 2000; 13:994-998.

5. Berthold H, Just A, Kirchheim HR, Ehmke H. Interactions Between Nitric Oxide and Endogenous Vasoconstrictors in Control of Renal Blood Flow. Hypertension 1999; 34:1254 -1258.
6. Bhagat K, Hingorami AD, Palacios M, Charles IG, Vallance P. Cytokine-induced venodilatation in humans in vivo: eNOS masquerading as iNOS. Cardiovascular Research 1999; 41:754-764.
7. Bilsborough W, Green DJ, Mamotte CD, van Bockxmeer FM, O'Driscoll GJ, Taylor RR. Endothelial nitric oxide synthase gene polymorphism, homocysteine, cholesterol and vascular endothelial function. Atherosclerosis, 2003; 169:131-138.
8. Bone RC, Grodzin CJ. Sepsis: A New Hypothesis for Pathogenesis of the Disease Process. Chest, 1997; 112: 235-243.
9. Bucher M, Ittner KP, Zimmermann M, Wolf K, Hobbhahn J, Kurtz A. Nitric oxide synthase isoform III gene expression in rat liver is up-regulated by lipopolysaccharide and lipoteichoic acid. FEBBS Lett 1997; 412: 511-514.
10. Castro L, Rodriguez M, Radi R. Aconitase is readily inactivated by peroxynitrite, but not by its precursor, nitric oxide. J Biol Chem, 1994; 269:29409-29415.
11. Colombo MG, Andreassi MG, Paradossi U, Botto N, Manfredi S, Masetti S, Rossi G, Clerico A, Biagini A. Evidence for association of a common variant of the endothelial nitric oxide synthase gene (Glu^{298}→Asp polymorphism) to the presence, extent, and severity of coronary artery disease. Heart, 2002; 87:525-528.
12. Colombo MG, Paradossi U, Andreassi MG, *et al.,*. Endothelial Nitric Oxide Synthase Gene Polymorphisms and Risk of Coronary Artery Disease. Clinical Chemistry, 2003; 49(3):389-395.

13. Doevendans PA, Jukema W, Spiering W, Defesche JC, Kastelein JJ. Molecular Genetics a gene expression in atherosclerosis. International Journal of Cardiology 2001; 80:161-172.
14. Fatini C, Sofi F, Gensini F, Sticchi E, Lari B, Pratesi G, Pulli R, Dorigo W, Pratesi C, Gensini GF, Abbate R. Influence of eNOS gene polymorphisms on carotid atherosclerosis. Eur. J. Vasc. Endovasc. Surg., 2004; 27:540-544.
15. Friedman G, Silva E, Vincent JL. Has the mortality of septic shock changed with time? Crit Care Med, 1998; 26:2078-2086.
16. Fujihara M, Muroi M, Tanamoto K, Suzuki T, Azuma H, Ikeda H. Molecular mechanisms of macrophage activation and deactivation by lipopolysaccharide: roles of the receptor complex. Pharmacol Ther, 100(2):171-94.
17. Gomez-Jimenez J, Salgado A, Mourelle M, Martin MC, Segura RM, Peracaula R, Moncada S. Nitric oxide pathway in endotoxemia and human septic shock. Crit Care Med, 1995; 23:253-257.
18. Granath B, Taylor RR, van Bockxmeer FM, Mamotte CD. Lack of evidence for association between endothelial nitric oxide synthase gene polymorphisms and coronary artery disease in the Australian Caucasian population. J Cardiovasc Risk, 2001; 8(4):235-241.
19. Haddad JJ, Fahlman CS. Nuclear Factor-κB- Independet Regulation of Lipopolyssaccharide- Mediated Interleukin- 6 Biosynthesis. Biochem and Biophysi Res Commu, 2002; 291:1045-1051.
20. Harkema JM, Dean RE, Stephan RN, Chaudry IH. Cellular dysfunction in sepsis. J Crit Care, 1990; 5:62-69.
21. Hibi K, Ishigami T, Tamura K, Mizushima S, Nyui N, Fujita T, Ochiai H, Kosuge M, Watanabe Y, Yoshii Y, Kihara M, Kimura K, Ishii M, Umemura S. Edothelial nitric

oxide synthase polymorphism and acute myocardial infarction. Hypertension, 1998; 32:521-526.

22. Hingorani AD, Liang CF, Fatibene J, Lyon A, Monteith S, Parsons A, Haydock S, Hopper RV, Stephens NG, O'Shaughnessy KM, Brown MJ. A common variant of the endothelial Nitric Oxide Sythase (Glu^{298}→Asp) is a major risk for coronary artery disease in the UK. Circulation, 1999; 100:1515-1520.
23. Hoesel LM, Ward PA. Mechanisms of inflammatory response syndrome in sepsis. Drug Discovery Today 2004; 3(1): 345 -350.
24. Hubacek JA, Stuber F, Frohlich D, Book M, Wetegrove S, Ritter M, Rothe G, Schmitz G. Gene variants of the bactericidal permeability creasing protein and lipopolysaccharide binding protein in sepsis patients: Gender-specific genetic predisposition to sepsis. Crit Care Med, 2001; 29:557-561.
25. Jo I, Moon J, Yoon S, Kim HT, Kim E, Park HY, Shin C, Min J, Jin YM, Cha SH, Jo SA. Interaction between -786TC polymorphism in the endothelial nitric oxide synthase gene and smoking for myocardial infarction in Korean population. Clin Chim Acta. 2006; 365(1-2):86-92.
26. Karima R, Matsumoto S, Higashi H, Matsushima K. The molecular pathogenesis of endotoxic shock. And organ failure. Molecular Medicine Today 1999; March 123-132.
27. Kilbourn R. Nitric oxide and shock. Dise Mon 1997; (43) 281 – 348.
28. Kim JU, Chang HK, Lee SS, Kim JW, Kim KT, Lee SW, Chung WT. Endothelial synthase nitric oxide polymorphisms in the Behçet´s disease and rheumatic diseases with vasculitis. Ann Rheum Dis, 2003; 62:1083-1087.
29. Knowles RG, Moncada S. Nitric oxide as a signal in blood vessels. Trends Biochem Sci, 1992; (17) 399-402.

30. Levecque C, Elbaz A, Clavel J, Richard F, Vidal JS, Amouyel P, Tzourio C, Alperovitch A, Chartier-Harlin MC. Association between Parkinson´s disease and polymorphisms in the nNOS and iNOS genes in a community –based case-control study. Human Molecular Genetics, 2003; 12(1):79-86.

31. Levy MM FM, Marshal JC, Abraham E, Angus D, Cook D, Cohen J, Opal SM, Vincent JL, Ramsay G. International Sepsis Definitions Conference, 2001 SCCM/ESCIM/ACCP/ATS/SIS International Sepsis Definitions Conference. Intensive Care Medicine 2003; 29(4):530-538.

32. López-Bojórquez LN, Dehesa AZ, Reyes-Terán G. Molecular Mechanisms Involved in the Pathogenesis of Septic Shock. Archives of Medical Research, 2004; 35: 465-479.

33. Medzhitov R. CpG DNA: security code for host defense. Nat Immunol, 2001; 2(1): 15-16.

34. Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991; 43:109-142.

35. Morisaki H, Sibbald WJ Tissue oxygen delivery and the microcirculation. Crit Care Clin. 2004; 20(2):213-223.

36. Naber CK, Baumgart D, Altmann C, Siffert C, Erbel R, Heusch G. eNOS 894T and coronary blood flow at rest and during adenosine-induced hyperemia. Am. J. Physiol. Heart. Circ. Physiol, 2001; 281: 1908-1912.

37. Netea MG, Meer JWM, Deuren M, Kulberg BJ. Proinflammatory cytokines and sepsis syndrome: not enough, or too much of a good thing? Trends in Immunology, 2003; 24(5) 254-258.

38. Noiri E, Satoh H, Taguchi J, Brodsky SV, Nakao A, Ogawa Y, Nishijima S, Yokomizo T, Tokunaga K, Fujita T. Association of eNOS Glu298Asp Polymorphism with End-Stage Renal Disease. Hypertension, 2002; 40: 535-540.

39. Novoradovsky A, Brantly ML, Waclawiw MA, Chaudhary PP, Ihara H, Qi L, Eissa NT, Barnes PM, Gabriele KM, Ehrmantraut ME, Rogliani P, Moss J. Edothelial Nitric Oxide Synthase as a Potential susceptibility gene in the pathogenesis of emphysema in α1-antytripsin deficiency. Am J Resp Cell Mol Biol 1999; 20: 441-447.
40. Rackow EC, Astiz ME. Pathophysiology and treatment of septic shock. JAMA, 1991; 266(4):548-554.
41. Salkowski CA, Detore C. McNally R, Rooijen N, Vogel SN. Regulation of inducible nitric oxide synthase messenger RNA expression and nitric oxide production by lipopolysaccharide in vivo – The roles of macrophages, endogenous IFN-gamma and TNF receptor-1-mediated signaling. J Immunol, 1997; (158) 905-912.
42. Sandrim VC, Coelho EB, Nobre F, *et al.,*. Susceptible and protective eNOS haplotypes in hypertensive black and white subjects. Atherosclerosis, 2006; 186:428–432.
43. Takeuchi O, Akira S. Tool like receptors: their physiological role and signal transduction system. Int Immunopharmacol, 2001:1(4):625-635.
44. Titheradge MA. Nitric oxide in septic shock. Biochimica et Biophysica Acta, 1999; 1411: 437-455.
45. Vincent JL: Sepsis: The magnitude of the problem In. The Sepsis Text 2002. Kluw er Academy Publisher.
46. Vincent JL, de Mendonca A, Cantraine F, Moreno R, Takala J, Suter PM, Sprung CL, Colardyn F, Blecher S.Use of the SOFA score to assess the incidence of organ dysfunction/failure in intensive care units: Results of a multicenter, prospective study. Crit Care Med, 1998; 26:1793-1800.
47. Wang JE, Jorgensen PF, Almlof M, Thiemermann C, Foster SJ, Aasen AO, Solberg R. Peptidoglycan and lipoteichoic acid from Staphylococcus aureus

induce tumor necrosis factor alpha, interleukin 6 (IL-6), and IL-10 production in both T cells and monocytes in a human whole blood model. Infect Immun, 2000; 68 (7):3965-3970.

JUSTIFICATIVA E OBJETIVO

A condição crítica de um paciente, a gravidade de suas disfunções orgânicas, a evolução para sepse, choque séptico ou para o óbito são características complexas determinadas pela interferência simultânea de centenas ou milhares de fatores externos e de fatores herdados. O estudo das variantes polimórficas do gene que codifica para a enzima que sintetiza constitutivamente o óxido nítrico no endotélio vascular (eNOS; sintase do óxido nítrico endotelial) pode ser útil na busca de desvendar parte do pequeno efeito que a herança genética pode ter no momento da doença crítica. Nosso objetivo neste trabalho foi, portanto, responder à pergunta que questiona se as variantes polimórficas -786T>C e 894G>T (Glu298Asp) do gene que codifica para a eNOS podem influenciar a susceptibilidade à sepse, ao choque séptico e às disfunções orgânicas em pacientes em condições críticas de saúde.

MANUSCRITO COM RESULTADOS DO TRABALHO EXPERIMENTAL

TITLE OF THE MANUSCRIPT

Single nucleotide polymorphisms of endothelial nitric oxide synthase (eNOS) and susceptibility to sepsis, septic shock and organ dysfunction.

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Index words:

eNOS polymorphism; -786C>T SNP; 894G>T SNP; genetic risk factors; sepsis; septic shock; organ dysfunction.

Periodic chosen for submission:

Intensive Care Medicine (ICM)

Abbreviations:

A - Adenine
AN - ANOVA test
APACHE-II - Acute Physiology and Chronic Health Evaluation II
Asp – Aspartate
C – Citosine
CD14 – Cluster of diferentiation 14
DNA - Deoxyribonucleic acid
eNOS – endothelial nitric oxide synthase
G - Guanine
Glu – Glutamate
Hosp – hospital
HSL - Hospital São Lucas
ICU - Intensive Care Unit
IFN-γ – interferon- γ
IL-1 - interleukin-1
LOS - length of stay
LPS - lipopolysaccharide
mRNA – Messenger ribonucleic acid
MW - mann-whitney *U*-test
nNOS – neuronal nitric oxide synthase
NO – nitric oxide
NOS – nitric oxide synthase
PCR - Polymerase chain reaction
PMNs – Polymorphonuclear neutrophils
PUCRS - Pontifícia Universidade Católica do Rio Grande do Sul
RFLP – Restriction Fragment Length Polymorphism
SD - standard deviation
SNP – Single nucleotide polymorphism
SNP - single nucleotide polymorphisms
SOFA - Sequential Organ Failure Assessment
SOFA1 - SOFA score at day 1, ICU admission
SOFA2 - SOFA score at day 2
SOFA3 - SOFA score at day 3
SOFA4 - SOFA score at day 4
SOFA5 - SOFA score at day 5
SOFA6 - SOFA score at day 6
SOFA7 - SOFA score at day 7
T - Timine
TLR - Toll-like receptor
TNF-α - tumor necrosis factor-alpha
χ^2 - pearson Chi-squared test

ABSTRACT

OBJECTIVE: To investigate the association between two single nucleotide polymorphisms (SNPs) of endothelial nitric oxide synthase (eNOS) and susceptibility to sepsis, septic shock and organ dysfunction in critically ill patients. PATIENTS AND METHODS: Two hundred and seven critically ill patients admitted to the intensive care unit (ICU) of São Lucas Hospital - RS, Brazil, from March 2002 to December 2005 were enrolled for study. 149 healthy volunteer DNA donors from the same population were genotyped control group. The organ dysfunction of septic patients was evaluated during the first week after ICU admission, and sepsis, septic shock, and mortality in ICU and hospital were considered. Genotypes of -786T>C and 894G>T (Glu298Asp protein alteration) SNPs were determined in patients and healthy controls by PCR-RFLP. Genotipic and allelic frequencies were analyzed. RESULTS: The frequency of -786C allele carriers in patients was significantly higher than those of healthy subjects (58% *vs* 46%; *P*=0.021, OR=1.64, $CI_{95\%}$: 1.05-2.57, and 60% *vs* 46%; *P*=0.012, OR=1.82, $CI_{95\%}$: 1.10-3.01, respectively). -786CC homozygote individuals had a significantly higher organ dysfunction measured by SOFA scores during first ICU week (*P*=0.001), although the presence of the -786C alelle was not related with different mortality rates. We detected more 894TT homozygotes among critically ill and septic shock patients as compared to healthy individuals (25% *vs* 14%; *P*=0.017, OR=0.49, $CI_{95\%}$: 0.25-0.92, and 25% *vs* 14%; *P*=0.024, OR=0.46, $CI_{95\%}$: 0.22-0.96, respectively). The 896G allele carriers had significantly higher SOFA scores to organ dysfunction (*P*=0.028), even though the inheritance of this allele did not affect the mortality. We found a significant higher presence of -786CC/894TT individuals among critically ill and septic patients than in healthy individuals (*P*=0.002), and the opposite double homozygote 786TT/894GG with higher frequency among healthy volunteers (*P*=0.002) if compared with patient group. CONCLUSION: Both the -786C>T and 894G>T eNOS SNPs may be associated with higher risk of susceptibility to development of critical clinical conditions, sepsis, and septic shock.

INTRODUCTION

Nitric oxide (NO) is an endogenous nitro-vasodilator regulated locally and the most basic and fundamental mechanism for adaptation of the vascular system [1,2]. This potent free radical vasodilator is produced from L-arginine by three different isoforms of NO synthase (NOS), two of which are expressed constitutively (eNOS in endothelium, nNOS in brain), while the third (iNOS) is induced in immunological system cells [3,4]. All three similar synthases consist of a heme domain, to which the substrate L-arginine binds, as well as tetrahydrobiopterin and Zn [5]. eNOS differs from iNOS in that its activity is dependent on the presence of calcium and calmodulin [5].

Constitutive proteins (eNOS and nNOS) are produced in response to physiological stimuli that trigger an intracellular Ca^{2+} signal; they synthesize NO rapidly and transiently at low concentrations [6]. Low-level NO produced by eNOS and nNOS is pro-inflammatory by inducing vasodilatation and the recruitment of neutrophils [6,7]. Constitutive endothelial NO, produced by eNOS in platelets and endothelial cells, plays a crucial role in the regulation of systemic vascular tone and in maintaining the functional and structural integrity of the vessel wall [8-10].

iNOS is not expressed in resting cells but is induced by immunological stimuli such as bacterial lipopolysaccharide (LPS) or cytokines such as IL-1, TNF-α, or IFN-γ, and produces NO at constant high levels [6]. At high concentrations it down-regulates adhesion molecules, suppresses activation, and induces apoptosis of inflammatory cells [7]. iNOS mRNA expression is activated in circulating polymorphonuclear neutrophils (PMNs) of patients with sepsis [4]; During sepsis and septic shock the excessive production of NO from iNOS gene over-expression,

following the cytokine-dependent induction, is essential to ensure the perfect immunological response [11], but it results in a progressive reduction of circulation flow to provide blood and oxygen to vital organs, producing an impaired tissue perfusion and oxygen extraction [12-14]. Septic patients have, consequently, organ dysfunction and failure attributable to a combination of excessive inflammation, disseminated coagulopathy, and disruption of integrity of micro vascular endothelium [11]. The hypotension and the vascular collapse present during septic shock may lead to critical organic condition, organ dysfunction or failure, and frequently death [12,14]. Sepsis followed by severe fall in blood pressure (septic shock) is a major cause of death among critically ill patients, with a mortality rate of 40-60%, despite treatment in intensive care units (ICU) [12].

At the same time that the favorable and unfavorable roles of iNOS expression in organ functions, sepsis, and endotoxic shock have being extensively documented [12-14], there remain substantial gaps in the understanding about the role of endothelial isoform of NOS in these critical clinical conditions. Even so, it is evident that changes on eNOS expression or activity might be detrimental or even lead to adverse effects [14,15].

A presumed alteration of eNOS would be explained, for instance, by reduction of the constitutive eNOS gene (*NOS3* human gene) expression or by an eNOS with changed functionality. In this way, two potentially functional cosegregating single nucleotide polymorphisms (SNPs) within the eNOS sequence could be crucial candidates to studies: -786T>C in the promoter region [16], and the guanine (G) to timine (T) transition at position 894 (894G>T) within of eNOS gene coding region [17]. While the first seems to reduce the transcriptional eNOS level [18], the last SNP cause a substitution in the amino acid sequence of eNOS protein (Glu298Asp

missense alteration) and seems to alter protein stability [19] and predispose to or accelerate coronary diseases [20-22].

Because during the organ dysfunction, sepsis, and septic shock the integrity of vascular endothelium can be affected by variations in eNOS expression or activity, we hypothesize that these genetic polymorphisms could be important factors in determining the clinical evolution and outcome in patients in such critical circumstances. Hence, in the present study, we investigate the -786T>C and 894G>T eNOS SNPs in patients from ICU population; we describe the frequency of these two genetics variations in different scores and degrees of organ failure, sepsis, and septic shock conditions. In addition, we correlate the genotype frequencies with the risk of critical clinical condition, sepsis, and septic shock.

PATIENTS AND METHODS

We studied 207 critically ill patients admitted to the General Intensive Care Unit (ICU) of the São Lucas Hospital (HSL), Brazil, between May 2002 and December 2005, and 149 healthy subjects DNA donors that were chosen serving as the normal control group. All patients and controls were from the southern Brazilian population. All patients or their answerable gave written, informed consent. Approval for human study protocols was obtained from the Research Ethics Committee of the Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS) (Protocol # 05/02598).

Patients Data.

A total of 207 critically ill adult patients (109 men and 98 women) admitted to the General Intensive Care Unit (ICU) at São Lucas Hospital – Pontifícia Universidade Católica do Rio Grande do Sul (HSL-PUCRS) were included in this study. We monitored the patients daily during their entire ICU and post-ICU (hospital) stay which resulted in measurements from the ICU admission day to a maximum of 224 days. Some critically ill adult patients were previously described partially by D'Avila *et al. 2006* [23]. Patients were not eligible if they were under 18 years old diagnosed with HIV-infection, pregnant, or lactating or taking immunosuppressive drugs.

For diagnosis of sepsis and septic shock we used the American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference criteria [24]: at least two of the following criteria: Fever or hypothermia (temperature in the core of the body > 38°C or < 36°C); Tachycardia (ventricular rate > 90 bpm); Tachypnea or Hyperventilation (> 20 breaths/min or $PaCO_2$ < 32 mmHg); Leucocytosis or Leucopenia.

For illness severity evaluation we used the APACHE-II (Acute Physiology And Chronic healthy Evaluation II) [25] score obtained on ICU admission day. The organ dysfunction and failure was evaluated using the SOFA (Sequential Organ Failure Assessment) score obtained on ICU admission day (SOFA-1) and during the first seven days following the ICU admission [26, 27]. Six different systems are evaluated regarding its clinical conditions to determine each SOFA score: cardiovascular function (systolic and diastolic blood pressure, use of vasopressors), liver function (serum bilirrubin levels), respiratory (PaO_2/FiO_2), neurological function (Glasgow coma score), coagulation function (platelet count), and renal function (serum creatinine levels, urine output). For each system, we considered without organ

dysfunction SOFA scores equal to zero, organ dysfunction scores 1 and 2, and organ failure scores 3 and 4. Total diary SOFA corresponds to a total of six systems SOFA scores.

Clinical endpoints of the study were discharge from the hospital or death. 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.

Genotyping of -798C>T and 896G>T eNOS SNPs:

A 5ml blood sample was collected in a sterile system with EDTA and maintained refrigerated at 4ºC or frozen at -20ºC until DNA extraction. Genomic DNA were isolated from leucocytes by standard procedures [28] and maintained at -20°C. Genotyping protocols for -786C>T and 894G>T (Glu298Asp) polymorphisms were previously described by Nakayama [16] and Nadaud [17], respectively. The 894G>T amplification was based in Hibi et al., 1998 [29]. Both DNA sequences were amplified in a 25 µL reaction containing 10-100 ng of DNA, 0.2 mmol/L of each dNTP, 1.5 mmol/L of $MgCl_2$ and 1.7 U Taq DNA Polymerase in Taq Buffer (Invitrogen-Life Technologies, São Paulo, Brazil). The -786C>T SNP was amplified using 10 pmol of each sense 5' TGG AGA GTG CTG GTG TAC CCC A 3'; and antisense 5' GCC TCC ACC CCC ACC CTG TC 3' primers (synthesized by Invitrogen-Life Technologies, São Paulo, Brazil) in a PTC-100 Thermocycler (MJ Research, Watertown, USA). The 894G>T SNP was similarly amplified using sense 5' TCC CTG AGG AGG GCA TGA GGC T 3' and antisense 5' TGA GGG TCA CAC AGG TTC CT 3' primers (synthesized by Invitrogen-Life Technologies, São Paulo, SP, Brazil) in the same thermocycler. Cycling conditions for both polymorphisms were 95°C for 5 min, followed by 36 cycles 94°C for 10 minutes, followed by 30 cycles at 94°C for 1 min, at 61°C for 1 min, and 72°C for 1 min. The final extension step was prolonged to 10

min. The PCR amplified products (20 µL) were cleaved, in an appropriate buffer, with 8-12U of *MspI* (5'C/CGG3'; New England Biolabs™, USA) for the -786C>T SNP; and 8-12U of the *Ban II* (5`GRGCY/C3` R= A or G and Y=C or T; New England Biolabs™, USA) for 896G>T SNP in a total volume of 25µL, at 37°C for 4 hours. The restriction on a 2% agarose gel, stained with ethidium bromide, and visualized over a UV light to determine the genotypes CC (90bp, 50bp and 40bp fragment); CT (140bp, 90bp, 50bp and 40bp); TT (140bp and 40bp) for -786C>T and GG (320bp and 137bp fragment); GT (457bp, 320bp and 137bp); TT (457bp) for 894G>T. The *Homo sapiens* nitric oxide synthase 3 (endothelial cell – NOS3) mRNA sequence is registered in the EMBL data base as GI:48762674 (GenBank accession number: NM_000603). Genotyping was performed in a blinded fashion, i.e., investigators were unaware of patient data.

Statistical analysis:

Statistical calculations were carried out using the statistical package SPSS 11.5 (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 Kruskal–Wallis and Mann–Whitney tests. For categorical data, we used Pearson Chi-squared test. To test Hardy–Weinberg equilibrium, the Chi-squared test was used. All reported P values are two-tailed, with 0.05 or less taken as significant.

RESULTS

Two hundred and seven critically ill patients were included in the study, 134 of them developed sepsis (64.7%, 134/207) and 93 of them had sepsis and septic

shock (44.9%, 93/207). The Table 1 illustrates a complete description of the 207 critically ill patient presented in three groups; all critically ill patients (n=207), all patients with sepsis (n=134), and only patients with septic shock (n=93). Dates of gender, age, APACHE II and SOFA-1 scores, organ dysfunction and failure, length of stay in ICU and post-ICU (hospital) can be visualized at Table 1. The same can be seemed to mortality rates.

We evaluate the influence of -786C>T and 894G>T SNPs in the same three patients groups mentioned above, in addition to 149 healthy subjects, to analyzed the genetic susceptibility to critical illness, sepsis, and septic shock (Figure 1). Figure 1-A shows that there were significant differences among rates of three -786C>T genotypes (-786CC, -786CT, -786TT) when we compared all critically ill patients and healthy subjects (Chi-square test, *P*=0.031), all septic patients and healthy subjects (Chi-square test, *P*=0.037), and only patients with septic shock and healthy subjects (Chi-square test, *P*=0.013). When studied simultaneously, the overall presence of -786CC homozygotes and -786CT heterozygotes was significantly higher in critically ill patients (58%; 120/207) than in healthy individuals (46%; 68/149) (*P*=0.021, OR=1.64, $CI_{95\%}$: 1.05-2.57), as well as in septic patients (60%; 81/134) than in healthy individuals (*P*=0.012, OR=1.82, $CI_{95\%}$: 1.10-3.01). Figure 1-B confirms statistical associations among rates of -786C>T alleles; correspondingly there was more -786C allele among septic patients (37%; 99/268) than in healthy subjects (29%; 86/298) (*P*=0.041, OR=1.44, $CI_{95\%}$: 1.00-2.09), as well as in septic patients (39%; 73/186) than in healthy individuals (*P*=0.018, OR=1.59, $CI_{95\%}$: 1.06-2.39).

Figure 1-C illustrates significant differences among rates of three 894G>T genotypes (894GG, 894GT, 894TT) when we contrasted all critically ill patients and the healthy subjects (Chi-square test, *P*=0.015), and septic patients and healthy subjects (Chi-square test, *P*=0.031). The global presence of 894TT homozygotes

was significantly higher inside critically ill patients group (25%; 51/207) than in healthy individuals group (14%; 17/149) when compared with 894GT heterozygotes and 894GG (*P*=0.017, OR=0.49, $CI_{95\%}$: 0.25-0.92). The same to septic shock patients group (25%; 24/93) against healthy individuals group (*P*=0.024, OR=0.46, $CI_{95\%}$: 0.22-0.96). The Figure 1-D specifies differences among rates of 894G>T alleles, but it has not statistical significance. To the entire evaluation presented in Figure 1 all statistical differences with P<0.05 was showed, and other associations did not find significant differences.

Finally, we analyzed the isolated effect of variants -786C, -786T, 894G, and 894T in critically ill, septic, and septic shock patients, as well as in the control group (Figure 2). In this evaluation we segregated only the four classes of double homozygotes: -786CC/894GG; -786CC/894TT; -786TT/894GG; -786TT/894TT. The Figure 2 shows that there were significant differences among rates of four double homozygotes when we compared all critically ill patients and the healthy subjects (Chi-square test, *P*<0.001), and all septic patients and the healthy subjects (Chi-square test, *P*<0.001). When we studied separately, the overall presence of -786CC/894TT was significantly higher in critically ill patients (15%; 31/207) than in healthy individuals (4%; 5/124) (*P*=0.002, OR=4.19, $CI_{95\%}$: 1.50-12.66), as well as in septic patients (15%; 20/134) than in healthy individuals (*P*=0.003, OR=4.18, $CI_{95\%}$: 1.42-13.18). In contrast, the presence of opposite double homozygote -786TT/894GG was significantly higher in healthy individuals (15%; 18/124) than critically ill patients (5%; 10/207) (*P*=0.002, OR=0.30, $CI_{95\%}$: 0.12-0.71), or in septic patients (5%; 7/134) (*P*=0.011, OR=0.32, $CI_{95\%}$: 0.12-0.86).

We found that total genotype and allele frequencies (patients and healthy subjects) to both SNPs were at Hardy-Weinberg equilibrium (-786C>T: CC=0.11, CT=0.42, TT=0.47 and C=0.32, T=0.68; Pearson Chi-square test *P*=0.819; 894G>T:

GG=0.24, GT=0.55, TT=0.21 and G=0.52, T=0.48; Chi-square test *P*=0.417). The isolated frequencies from patients' or healthy volunteers' samples did not differ from the values expected by the Hardy–Weinberg model too.

The effect of -786C>T and 894G>T SNPs was estimated during the first ICU week in order to analyze the genetic susceptibility in the course of critical clinical condition, in organ dysfunction, and in organ failure (Figure 3). Figure 3-A shows that among -786C>T genotypes there was a significant difference in SOFA scores throughout the first ICU week; -786CC homozygotes present higher SOFA score means (7.67±0.41) when compared to -786CT (6.31±0.22) and -786TT (6.17±0.31) (Kruskal-Wallis test, *P*=0.001). Figures 3-B illustrate the genotypes frequencies to -786C>T SNP examining organ condition of the six systems during a first week from ICU admission. We consider two categories; organ dysfunction when system SOFA scores were only 1 or 2, and organ failure when they were, at least, 3 or 4. In this evaluation none significant association was found (Pearson Chi-square test, *P*=0.969), that explicit there was similar rates of organ dysfunction and failure among genotypes. Figure 3-C demonstrates that we found statistical association among 894G>T genotypes; SOFA means were higher in the 894GG homozygotes and 894GT heterozygotes group (6.68±0.30) when compared with 896TT (6.02±0.48) (Kruskal-Wallis test, *P*=0.028). Figure 3-D shows the genotype frequencies to 894G>T SNP without significant association (Pearson Chi-square test, *P*=0.470).

The frequencies of -786C>T and 894G>T genotypes were determined considering the mortality rates. We evaluate the effect of SNPs in the three patients groups: all critically ill patients (n=207), all septic patients (n=134), only patients with septic shock (n=93). Figure 4 demonstrates that there were no significant differences among -786C>T (Figure 4-A) and 894G>T (Figure 4-B) genotypes in the mortality rates of patients monitored daily during their entire ICU and post-ICU (hospital) stay

from the ICU admission to a maximum of 224 days. The *P* values are shown in the figure legend. Hospital deaths showed similar results; -786CC (63%, 14/22), -786CT (41%, 40/98), -786TT (49%, 43/87) (Chi-square test, *P*=0.080), and 894GG (48%, 28/54), 894GT (48%, 49/102), 894TT (41%, 21/51) (Chi-square test, *P*=0.678).

DISCUSSION

The excessive production of NO in organ dysfunction, sepsis, and endotoxic shock is especially well known from iNOS gene induction [12-14]; however there is a modest knowledge about the effect of endothelial isoform of NO syntase (eNOS) during these critical clinical conditions. Therefore, the aim of our study was to supply information to contribute in the understanding of the role of eNOS.

We evaluated the influence of the -786C>T and 894G>T eNOS SNPs in critical ill patients admitted on intensive care unit (ICU) and contrasted with healthy volunteers. Our sample from southern Brazil, mainly of European ancestry (descendants from Italian, German, and Portuguese origins) was large (n=356 individuals) and homogeneous since the genotype and allele frequencies were at Hardy–Weinberg equilibrium. When compared to previous published studies from different populations, our -786C>T and 894G>T genotype and allele frequencies were more similar to populations with European ethnic component than to Asiatic populations (Table 2). However, the frequency for 894T allele (896T=0.48) was more elevated if compared to those reported by authors who also analyze subjects from southern Brazil [894T=0.36 (30); 894T=0.15 (31)] (Table 2).

We found significantly higher frequency of -786C allele carriers among critically ill and septic patients when compared with healthy subjects. The same allele was associated significantly with higher organ dysfunction measured by SOFA

scores during first ICU week, although -786C carriers was not related with different mortality rates. We found evidence that polymorphic -786C eNOS variant can be independently and significantly associated with elevated risk to reach critical clinical and septic conditions. In parallel, we detected more 894TT homozygotes among critically ill and with septic shock patients in contrast with healthy individuals. Surprisingly, the 894G, but not 894T, allele carriers had significantly higher SOFA scores to organ dysfunction, even though the inheritance of this allele did not affect the mortality. We interpreted this result considering that 894TT homozygotes have greater risk to reach critical clinical and septic shock conditions; however when a patient is critically ill the isled influence of 894G allele is detrimental and can lead to adverse effects. The conflicting effect of 894G>T SNP in organ dysfunction also can be explain by a cumulative effect of two SNPs or because that this polymorphism is in linkage disequilibrium with other functional variants within eNOS gene (e.g. polymorphic variation into intron 4) or another gene.

The -786C>T eNOS SNP was originally reported in patients with coronary vasospasm which -786C variant decreased promoter activity by ≅50%, suggesting that the L-arginine/NO pathway does not function correctly in -786C carriers [16, 32]. Indeed, studies in southern Brazilian population confirm that -786C allele predispose to coronary artery disease (CAD) [33], and can indicate a tendency for development of severe clinical course of sickle cell disease [31]. Barbosa F Jr *et al.,* 2004 supported that this promoter variant may be especially relevant for the development of CAD in Caucasians [18].

The 894T allele may predispose to CAD [21], hypertension [33], atherosclerosis [34], or stroke [35], suggesting that 894TT homozygote or 894T carriers could produce a significant decrease in amount of eNOS or enzymatic activity. This relationship between pathological phenotype and 894G>T SNP has

been strict but still inconsistent. While it was reported that eNOS protein with aspartic acid in position 298 (resultant from 894T allele expression) has enhanced susceptibility to intracellular proteolytic cleavage in endothelial cell lines compared with eNOS protein with glutamic acid (894G allele) [36], this substitution does not seem modulate eNOS activity *in vivo* [37]. Likewise, it has been reported that neither -786C>T or 894G>T eNOS SNPs significantly influenced plasma nitrite / nitrate concentrations and the risk of ischemic heart disease in a large cohort of middle-aged men [36]. However, the functional importance of the diminished eNOS expression was reveled by the finding that serum nitrite / nitrate levels among individuals carrying the -786C allele were significantly lower than among those without this variant [19].

Even there were a largest conflicting findings, -786C>T and 894G>T eNOS SNPs are important genetic markers and it is necessary to explore and clarify their putative effects in endothelial NO function.

Our data showed a significant higher presence of -786CC/894TT among critically ill and septic patients than in healthy individuals, and the opposite double homozygote 786TT/894GG with higher frequency among healthy volunteers if compared with patient group. Looking for a single interpretation to inheritance of two polymorphic variants in our sample, we can consider that if is proper the proposition that -786C and 894T alleles interfere respectively in the synthesis and activity of eNOS, we could explain our data supporting that patients with -786C and 894T alleles have greater susceptibility to attain critical state because the inhibition of the constitutively expressed isoform of NOS, which is essential to maintain organ perfusion, may be detrimental [38], starting to be an important linked co-morbidity factor.

Regularly large quantities of NO produced by both eNOS and iNOS may be beneficial to reduced the tissue damage associated with sepsis, septic shock and organ dysfunction septic shock because it would reduce the tissue microcirculatory hypoperfusion that is a dominant factor to these pathogenesis [12, 39]. Since iNOS is not expressed homogeneously in organ systems, areas lacking iNOS have less NO-induced vasodilation and become underperfused [40]. In this case NO produced by eNOS in regularly large quantities may be fundamental. Microcirculatory function is the main prerequisite for adequate tissue oxygenation and thus organ function. Its purpose is to transport oxygen and nutrients to tissue cells, ensure adequate immunological function and, in disease, to deliver therapeutic drugs to target cells. Thus, the microcirculatory mechanisms regulated by endothelium is a defining factor in the pathophysiology of sepsis [41].

Because -786C>T and 894G>T SNPs seem directly to interfere in or to be in linkage disequilibrium with the eNOS differential function, affecting in consequence the integrity of vascular endothelium, the study of these genetic variants can explain, at least in part, the patients susceptibility to sepsis, septic shock and severe organ dysfunction. The presence of different haplotype arrangements of eNOS SNPs may thus be predictive in an intensive care unit to the patients' outcome.

The pathogenesis of sepsis, septic shock, and organ dysfunction are multifactorial and incompletely understood. The identifying of more homogeneous genetic subgroups of patients may be a fine strategy to study specifically targeted therapeutic interventions. Based on the present and previous observations, we propose that -786C>T and 894G>T eNOS SNPs can be considered as a potential prognostic genetic marker for sepsis, shock septic, and severe organ dysfunction, and an independent and significant risk factor to critical clinical and septic conditions.

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REFERENCES

1. Marín J, Rodriguez-Martinez MA (1997) Role of vascular nitric oxide in physiological and pathological conditions. Pharmacol Ther 75: 111-134.
2. Moncada S, Palmer RMJ, Higgs EA (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 43:109-142.
3. Moncada S, Higgs A (1993) The L-arginine-nitric oxide pathway. N Engl J Med 329:2002–2012.
4. Tsukahara Y, Morisaki T, Horita Y, Torisu M, Tanaka M (1998) Expression of Inducible Nitric Oxide Synthase in Circulating Neutrophils of the Systemic Inflammatory Response Syndrome and Septic Patients. World J Surg 22:771–777.
5. Govers R, Rabelink TJ (2001) Cellular regulation of endothelial nitric oxide synthase. Am J Physiol Renal Physiol 280:193-206.
6. Coleman JW (2001) Nitric oxide in immunity and inflammation. Int Immunopharmacol 1: 1397–406.
7. Netea MG, Meer JWM, Deuren M, Kulberg BJ (2003) Proinflammatory cytokines and sepsis syndrome: not enough, or too much of a good thing? Trends Immunol 24(5): 254-258.

8. Loscalzo J (2001) Nitric oxide insufficiency, platelet activation, and arterial thrombosis. Circ Res 88:756–752.
9. Ignarro LJ (1989) Endothelium-derived nitric oxide: actions and properties. *FASEB J* 3:31–36.
10. Förstermann U, Boissel JP, Kleinert H (1998) Expressional control of the 'constitutive' isoforms of nitric oxide synthase (NOS I and NOS III). FASEB J 12:773–790.
11. Cohen J (2002) The immunopathogenesis of sepsis. Nature 420:885-891.
12. Titheradge MA (1999) Nitric oxide in septic shock Biochim Biophys Acta (1411) 437-455.
13. Szabo C, Thiemermann C (1994) Invited opinion: Role of nitric oxide in hemorrhagic, traumatic, and anaphylactic shock and thermal injury. *Shock* 2:145–155.
14. Thiemermann C (1997) Nitric oxide and septic shock. Gen Pharmac 29:159–166.
15. Kim JU, Chang HK, Lee SS, Kim JW, Kim KT, Lee SW, Chung VT (2003) Endothelial synthase nitric oxide polymorphisms in the Behçet´s disease and rheumatic diseases with vasculitis. Ann Rheum Dis 62:1083-1087.
16. Nakayama M, Yasue H, Yoshimura M, Shimasaki Y, Kugiyama K, Ogawa H, Motoyama T, Saito Y, Ogawa Y, Miyamoto Y, Nakao K (1999) $T^{-78\ 6} \rightarrow$ C Mutation in the 5'-Flanking Region of the Endothelial Nitric Oxide Synthase Gene Is Associated With Coronary Spasm. Circulation 99: 28-64-2870.
17. Nadaud, S., Bonnardeaux, A., Lathrop, M., and Soubrier, F.(1994) Gene structure, polymorphism and mapping of the human endothelial nitric oxide synthase gene. *Biochem Biophys Res Commun* 198:1027–1033.

18. Barbosa F Jr, Sandrim VC, Uzuelli JA, Gerlach RF, Tanus-Santos JE (2004) eNOS genotype-dependent correlation between whole blood lead and plasma nitric oxide products concentrations. Nitric Oxide 14(1):58-64
19. Tesauro M, Thompson WC, Rogliani P, Qi L, Chaudhary PP, Moss J (2000) Intracellular processing of endothelial nitric oxide synthase isoforms associated with differences in severity of cardiopulmonary diseases: cleavage of proteins with aspartate vs. glutamate at position 298. *Proc Natl Acad Sci U S A* 97:2832–2835.
20. Gardemann A, Lohre J, Cayci S, Katz N, Tillmanns H, Haberbosch W (2002) The T allele of the missense Glu298Asp endothelial nitric oxide synthase gene polymorphism is associated with coronary heart disease in younger individuals with high atherosclerotic risk profile. Atherosclerosis 160:167–175.
21. Colombo M.G, Andreassi MG, Paradossi U, Botto N, Manfredi S, Masseti S, Rossi G, Clerico A, Biagini A (2002) Evidence for association of a common variant of the endothelial nitric oxide synthase gene (Glu^{298}→Asp polymorphism) to the presence, extent, and severity of coronary artery disease. Heart 87: 525-528.
22. Hingorami AD, Liang CF, Fatibene J, Lyon A, Monteith S, Parsons A, Haydock S, Hooper RV, Stephens NG, O'Shaugnessy KM, Brown MJ (1999) A common variant of the endothelial Nitric Oxide Sythase (Glu^{298}→Asp) is a major risk for coronary artery disease in the UK. Circulation 100:1515-1520.
23. D'Avila LC, Albarus MH, Franco CR, Aguiar BB, Oliveira JR, Dias FS, Alho CS (2006) Effect of CD14 -260C>T polymorphism on the mortality of critically ill patients. Immunol Cell Biol 4:342-348
24. Levy MM FM, Marshal JC, Abraham E, Angus D, Cook D, Cohen J, Opal SM, Vincent JL, Ramsay G. (2003) International Sepsis Definitions Conference,

2001 SCCM/ESCIM/ACCP/ATS/SIS International Sepsis Definitions Conference. Int Care Med 29(4):530-538.

25. Knaus WA, Draper EA, Wagner DP, Zimmerman JE (1985) APACHE II: a severity of disease classification system. Crit Care Med 13(10):818-829.
26. Vincent JL, de Mendonca A, Cantraine F, Moreno R, Takala J, Suter PM, Sprung CL, Colardyn F, Blecher S (1998) Use of the SOFA score to assess the incidence of organ dysfunction/failure in intensive care units: Results of a multicenter, prospective study. Crit Care Med 26:1793-1800.
27. Ferreira FL, Bota DL, Bross A, Mélot C, Vincent JL (2001) Serial evaluation of the SOFA score to predict outcome in critically ill patients JAMA 286(14):1754-1758.
28. Lahiri DK, Nurnberger Jr JI (1991) A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. Nucl Acids Res 19: 5444.
29. Hibi K, Ishigami T, Tamura K, Mizushima S, Nyui N, Fujita T, Ochiai H, Kosuge M, Watanabe Y, Yoshii Y, Kihara M, Kimura K, Ishii M, Umemura S (1998) Endothelial nitric oxide synthase polymorphism and acute myocardial infarction. Hypertension 32:521-526.
30. Iturry-Yamamoto GR, Moriguchi EH, Zago AC, Alho CS, Zago AJ (2007) Association of the 894G>T polymorphism of endothelial constitutive nitric oxide synthase gene with unstable angina. Braz J Med Biol Res (Epub ahead of print).
31. Vargas AE, Silva MAL, Silla L, Chies JAB (2005) Polymorphisms of chemokine receptors and eNOS in Brazilian patients with sickle cell disease. Tissue Antigens 66:683-690.

32. Rios DL, Callegari-Jacques SM, Hutz MH (2005). Endothelial nitric oxide synthase and fractalkine chemokine receptor polymorphisms on angiographically assessed coronary atherosclerosis. Clin Chim Acta 362:138-146.

33. Miyamoto Y, Saito Y, Kajiyama N, Yoshimura M, Shimasaki Y,, Nakayama M, Kamitani S, Harada M, Ishikawa M, Kuwahara K, Ogawa E, Hamanaka I, Takahashi N, Kaneshige T, Teraoka H, Akamizu T, Azuma N, Yoshimasa Y, Yoshimasa T, Itoh H, Masuda I, Yasue H, Nakao K (1998) Endothelial nitric oxide synthase gene is positively associated with essential hypertension. Hypertension 32:3–8.

34. Fatini C, Sofi F, Gensini F, Sticchi E, Lari B, Pratesi G, Pulli R, Dorigo W, Pratesi C, Gensini GF, Abbate R. (2004) Influence of eNOS gene polymorphisms on carotid atherosclerosis.Eur J Vasc Endovasc Surg 27(5):540-544.

35. Casas JP, Bautista LE, Humphries SE, Hingorani AD (2004). Endothelial nitric oxide synthase genotype and ischemic heart disease: meta-analysis of 26 studies involving 23028 subjects. *Circulation* 109: 1359 –1365.

36. Jeerooburkhan N, Jones LC, Bujac S, Cooper JA, Miller GJ, Vallance P, Humphries SE, Hingorani AD (2001). Genetic and environmental determinants of plasma nitrogen oxides and risk of ischemic heart disease. Hypertension 38(5):1054-1061.

37. McDonald DM, Alp NJ, Channon KM (2004) Functional comparison of the endothelial nitric oxide synthase E298D polymorphic variants in human endothelial cells. Pharmacogenetics 14: 831–839.

38. Fukatsu K, Saito H, Fukushima R, Inoue T, Lin MT, Inaba T, Muto T (1995). Detrimental effects of a nitric oxide synthase inhibitor (N-omega-nitro-L-arginine-methyl-ester) in a murine sepsis model. Arch Surg 130(4):410-414.
39. Cohen J (2002) The immunopathogenesis of sepsis. Nature 420:885-891.
40. Spronk PE, Zandstra DF, Ince C (2004) Bench-to-bedside review: Sepsis is a disease of the microcirculation. Crit Care Med 8:462-468.
41. Ince C (2005) The microcirculation is the motor of sepsis. Crit Care Med 9 Suppl 4:S13-9. Epub.

Table 1. Demographic and clinical characteristics of patients admitted in intensive care unit.

	Patients admitted in ICU		
	All Critically ill	**All septic**	**Septic shock**
Frequency [N (%)]	207 (100)	134 (64.7)	93 (44.9)
Female [N (%)]	98 (47.3)	61 (45.5)	40 (43.0)
Age [mean (SD)]	54.8 (19.7)	56.0 (18.4)	57.3 (17.2)
APACHE II Score [mean (SD)]	20.23 (7.8)	21.7 (7.1)	21.97 (6.9)
SOFA-1 [mean (SD)]	6.56 (3.4)	7.52 (3.4)	8.55 (3.1)
With only organ dysfunction [N (%)]	20 (9.7)	12 (8.9)	9 (9.7)
With organ failure [N (%)]	187 (90.3)	122 (91.0)	84 (90.3)
ICU LOS [median (min/max)]	13 (0/82)	15 (0/82)	15 (0/82)
ICU+H LOS [median (min/max)]	35 (4/224)	36 (6/122)	36 (6/118)
Mortality in ICU [N (%)]	73 (35.3)	67 (50.0)	58 (62.4)
Mortality in ICU+H [N (%)]	97 (46.8)	78 (58.2)	63 (67.7)

Two hundred and seven critically ill patients included in the study presented in three groups: all critically ill patients (n=207), all septic patients of them (n=134), and only patients with septic shock (n=93). N, number of patients; SD, standard deviation of the mean; ICU: Intensive care unit; APACHE II: Acute Physiology And Chronic healthy Evaluation II score obtained on ICU admission day; SOFA-1: Sequential Organ Failure Assessment score obtained daily during the first week from the ICU admission (day 1); Organ dysfunction: when six systems SOFA scores were only 1 or 2 during a first ICU week; Organ failure: when six systems SOFA scores were, at least once, 3 or 4 during a first ICU week. ICU LOS: Length of stay at intensive care unit, measured by number of days; ICU+H LOS: Total length of stay at ICU and post-ICU (hospital), measured by number of days; Mortality: outcome of patients in total length of stay at ICU and ICU+H.

Table 2: Characteristics of studies reporting -786C>T (A) and 894G>T (B) allele frequencies in different populations.

(A) Author, Year	n	-786C allele	-786T allele	Local
Nakayama *et al.,* 2000	554	0.09	0.91	Japan
Kim *et al.,* 2006	369	0.10	0.90	Korea
Jia *et al.,* 2006	402	0.13	0.87	China
Sandrin *et al.,* 2006b	178	0.28	0.72	black subjects - Brazil
Sampaio *et al.,* 2006	232	0.31	0.69	São Paulo, Brazil
This study, 2007	356	0.32	0.68	Rio Grande do Sul, Brazil
Syllos *et al.,* 2006	273	0.34	0.66	Brazil
Sandrin *et al.,* 2006a	400	0.34	0.66	Brazil
Sandrin *et al.,* 2006b	225	0.40	0.60	white subjects - Brazil
Alvarez *et al.,* 2000	470	0.40	0.60	Spain
Ordonez *et al.,* 2000	404	0.41	0.59	Spain
Fatini *et al.,* 2003	1014	0.43	0.57	Italy
Rossi *et al.,* 2003	185	0.46	0.54	Italy

(B) Author, Year	n	894G allele	894T allele	Local
This study, 2007	331	0.52	0.48	Rio Grande do Sul, Brazil
Hingorani *et al.,* 1999	868	0.63	0.37	England
Fatini *et al.,* 2003	1014	0.64	0.36	Italy
Yamamoto *et al.,* 2007	156	0.64	0.36	Rio Grande do Sul, Brazil
Rossi *et al.,* 2003	185	0.65	0.35	Italy
Markus *et al.,*1998	597	0.65	0.35	England
MacLeod *et al.,*1999	658	0.65	0.35	Scotland
Poirier *et al.,* 1998	1107	0.66	0.34	France / Northern Ireland
Jachymova *et al.,* 2001	323	0.67	0.33	Prague, Czech Republic
Elbaz *et al.,* 2000	920	0.67	0.33	France
Sandrin *et al.,* 2006b	225	0.69	0.31	white subjects - Brazil
Sampaio *et al.,* 2006	232	0.70	0.30	São Paulo, Brazil
Sandrin *et al.,* 2006a	400	0.71	0.29	Brazil
Syllos *et al.,* 2006	273	0.72	0.28	Brazil
Sandrin *et al.,* 2006b	178	0.76	0.24	black subjects - Brazil
Vargas *et al.,* 2005	173	0.85	0.15	Rio Grande do Sul, Brazil
Kim *et al.,* 2006	369	0.90	0.10	Korea
Shoji *et al.,* 2000	376	0.90	0.10	Japan
Yoshimura T *et al.,* 2001	205	0.90	0.10	Japan
Hibi *et al.,* 1998	583	0.91	0.09	Japan
Yoshimura T *et al.,* 2000	322	0.91	0.09	Japan
Yoshimura *et al.,*1998	213	0.92	0.08	Japan
Shimasaki *et al.,* 1998	892	0.92	0.08	Japan
Miyamoto *et al.,* 1998	868	0.92	0.08	Japan
Kato *et al.,* 1998	1062	0.92	0.08	Japan
Yoshimura M *et al.,* 2000	546	0.92	0.08	Japan

n: number total of studied subjects (cases + controls).

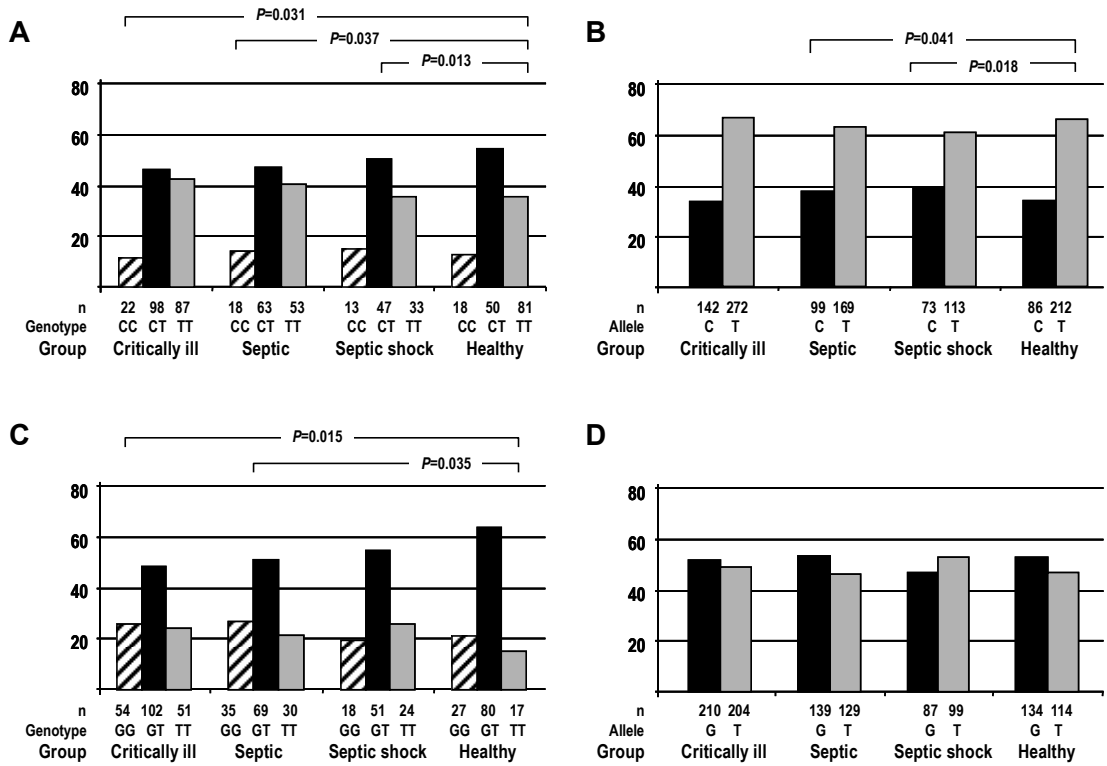

Figure 1. -786C>T and 894G>T SNPs frequencies in the three patient categories and in healthy subjects. A and B: Percentiles of -786C>T genotypes and alleles, respectively, of critically ill patients (n=207), septic patients (n=134), patients with septic shock (n=93), and in healthy subjects (n=149). C and D: Percentiles of 894G>T genotypes and alleles, respectively, of critically ill patients (n=207), septic patients (n=134), patients with septic shock (n=93), and in healthy subjects (n=124). Genotypic and allele frequencies are indicated as percents and numbers (n). Genotype and allele classes are indicated below the colored bars. Statistical associations were calculated by Chi-square test. Only the significant differences are shown ($P<0.05$). Other comparison found no significant differences.

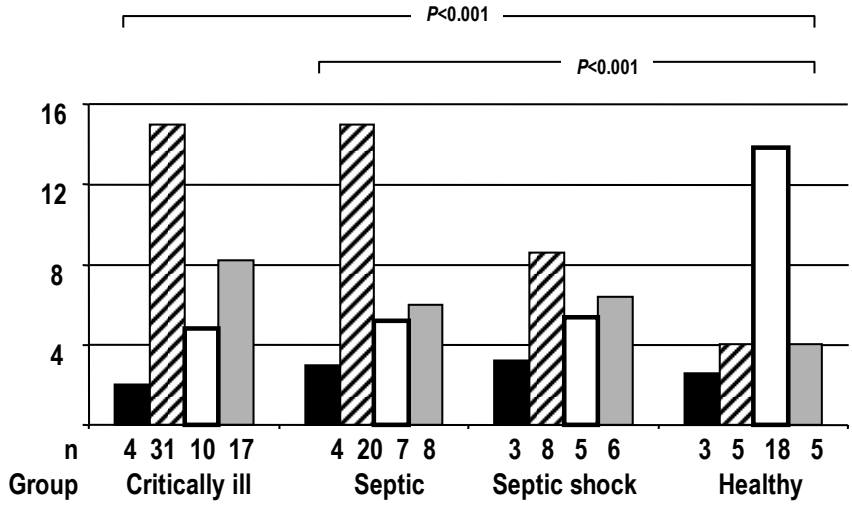

Figure 2. Percentiles of double homozygote classes in the three patients' categories and in healthy subjects. The four classes of double homozygotes are colored as: -786CC/894GG: black; -786CC/894TT: striped; -786TT/894GG: white; -786TT/894TT: gray. All critically ill patients (n=207), all septic patients (n=134), only patients with septic shock (n=93), healthy subjects (n=124). Frequencies are indicated as percents and numbers (n). Statistical associations were calculated by Chi-square test. Only the significant differences are shown (P<0.05). Other comparison found no significant differences.

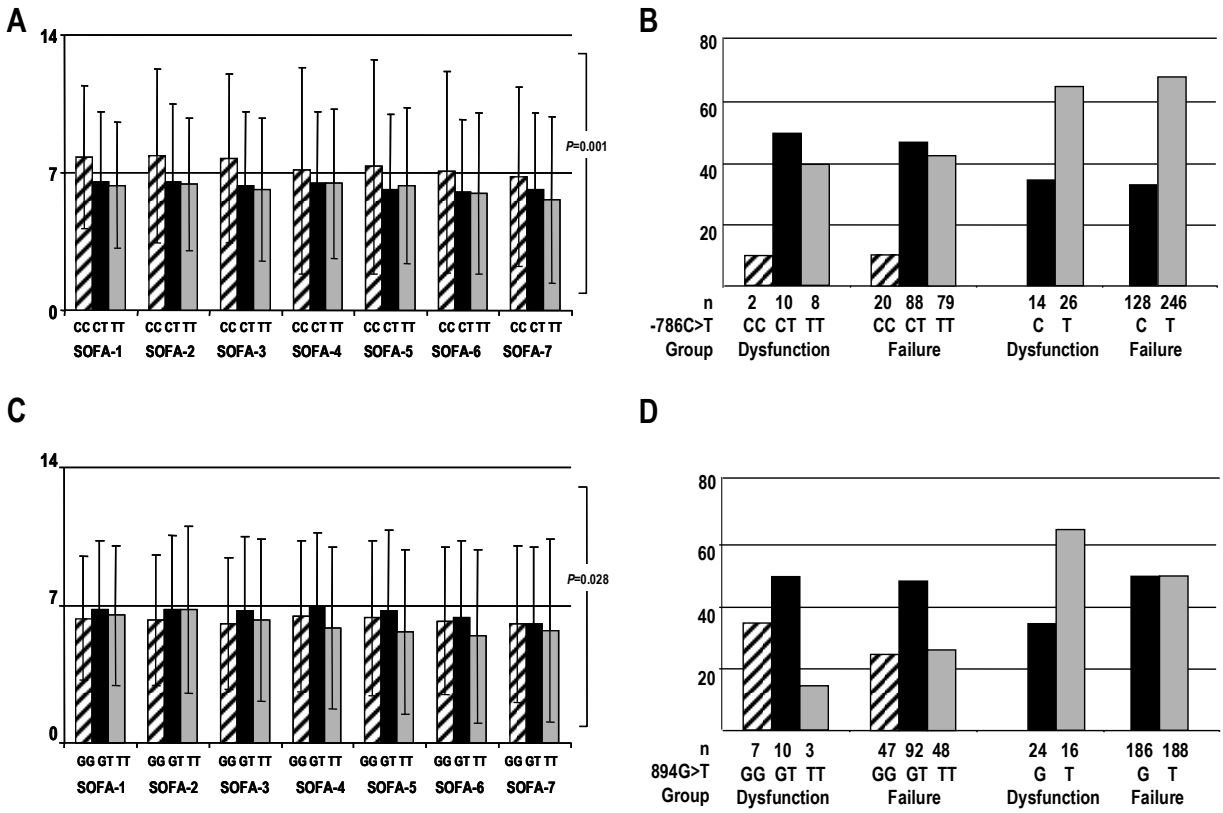

Figure 3. Means of SOFA scores, organ dysfunction, and organ failure frequencies by -786C>T and 894G>T genotypes during a first ICU week. A: Mean and standard deviation of daily SOFA score by -786C>T genotypes. B: organ dysfunction and organ failure frequencies by -786C>T genotypes. C: Mean and standard deviation of daily SOFA score by 894G>T genotypes. D: organ dysfunction and organ failure frequencies by 894G>T genotypes. Dysfunction: patients with only daily SOFA scores equal to 1 or 2 during ICU first week stay. Failure: patients with any SOFA scores equal to 3 or 4 during ICU first week stay. Statistical associations were calculated in A and B by Kruskal-Wallis test, and in C and D by Chi-square test. Genotypic and allele frequencies are indicated as percents and numbers (n). Genotype and allele classes are indicated below the colored bars. Only the significant differences are shown (P>0.05). Other comparison found no significant differences.

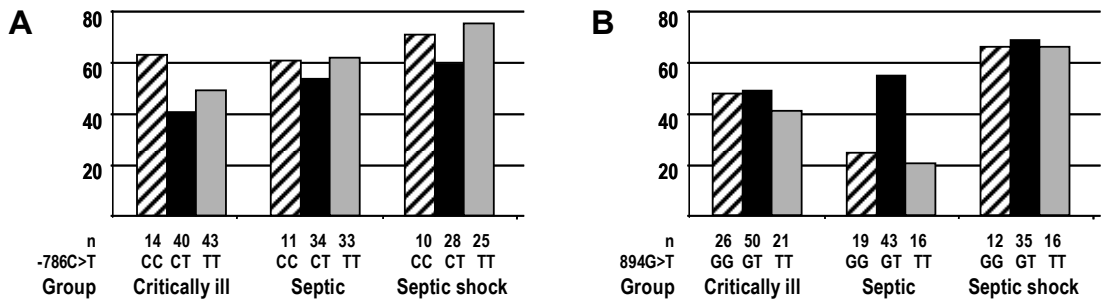

Figure 4. Mortality rates by -786C>T and 894G>T SNPs genotypes. A: Percentiles of deceased by -786C>T genotypes of all critically ill patients (n=207; *P*=0.080), all septic patients (n=134; *P*=0.574), only patients with septic shock (n=93; *P*=0.234). B: Percentiles of deceased by 894G>T genotypes of all critically ill patients (n=207; *P*=0.678), all septic patients (n=134; *P*=0.545), only patients with septic shock (n=93; *P*=0.980). Genotypic and allele frequencies are indicated as percents and numbers (n). Genotype and allele classes are indicated below the colored bars. Statistical associations were calculated by Chi-square test.

CONSIDERAÇÕES FINAIS

A condição crítica de um paciente, a gravidade de suas disfunções orgânicas, a evolução para sepse, para o choque séptico ou para o óbito são características complexas determinadas pela interferência de múltiplos fatores. Centenas ou milhares de fatores externos e de fatores intrínsecos determinarão simultaneamente a susceptibilidade para e o desfecho de uma condição clínica patológica crítica. Cada fator externo, e cada um dos genes herdados, exercerá isoladamente um pequeno efeito, mas que cumulativamente, no somatório com os demais, definirá a evolução do quadro clínico. O estado de saúde, o prognóstico e o desfecho de pacientes em condições críticas de saúde estão, portanto, também relacionados à herança genética que indivíduo recebeu.

Ainda que centenas de genes estejam envolvidos na modulação fisiológica final de um indivíduo com um quadro patológico complexo, aqueles genes que interferem em múltiplos sistemas e órgãos são sempre muito decisivos. Trabalhar com as variantes polimórficas do gene que codifica para a enzima que sintetiza constitutivamente o óxido nítrico no endotélio vascular (eNOS; sintase do óxido nítrico endotelial) foi um esforço na busca de desvendar parte do pequeno efeito que a herança genética pode ter no momento da doença crítica.

Como apresentado ao longo deste trabalho, nosso objetivo foi responder à pergunta que questiona se variantes polimórficas do gene que codifica para a eNOS poderiam influenciar a susceptibilidade à sepse, ao choque séptico e às disfunções orgânicas em pacientes críticos. Nós verificamos que, pelo menos em parte, a susceptibilidade à sepse ao choque séptico e à gravidade das disfunções orgânicas pode ser decorrente da herança das variantes polimóficas da eNOS.

Baseado em registros prévios, pode-se considerar que as variantes polimóficas -786C>T e 894G>T estão ligadas ou interferem diretamente na função diferencial da eNOS e isso afeta, consequentemente, a manutenção da integridade do endotélio vascular. Sendo assim, nossos resultados mostram que a herança diferencial de alelos destes SNPs (*single nucleotide polymorphism*) pode ser, de fato, um fator de risco independente e significante de susceptibilidade para a gravidade da condição clínica crítica, para a evolução da sepse e do choque séptico. A presença de diferentes arranjos haplotípicos pode ser também preditiva no desfecho de pacientes internados em uma unidade de terapia intensiva.

Em conclusão, baseado-nos em observações prévias e no presente estudo, nós propomos que as variantes polimórficas -786C>T e 894G>T do gene que codifica para a eNOS podem ser considerados potenciais marcadores genéticos para a sepse, choque séptico e para gravidade das disfunções orgânicas em pacientes em estado crítico de saúde.