FACULDADE DE BIOCIÊNCIAS
Programa de Pós-Graduação em Biologia Celular e Molecular
DOUTORADO

PAULO ROBERTO VARGAS FALLAVENA

VARIANTES POLIMÓRFICAS DOS GENES QUE CODIFICAM O CD14, TLR2, TLR4 E TNF-α ENVOLVIDOS COM O PROCESSO INFLAMATÓRIOEM PACIENTES EM CONDIÇÕES CRÍTICAS DE SAÚDE

Porto Alegre
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Tese apresentada ao Programa de Pós-Graduação em Biologia Celular e Molecular da PUCRS como requisito parcial e último para a obtenção do título de Doutor em Biologia Celular e Molecular.

Orientadora: Clarice Sampaio Alho

Porto Alegre
Maio 2011

DEDICATÓRIA

**Dedico esta Tese aos meus Pais,
que sempre estiveram ao meu lado, Minha namorada Thayse pelo amor e suporte nas horas difíceis, a todos que trabalharam comigo e fizeram este estudo possível, e a minha orientadora Clarice Alho, pela dedicação, suporte e brilhante orientação.**

AGRADECIMENTOS

Agradeço a todos que passaram pelo Laboratório de Genética Humana pelo apoio, suporte técnico, aos meus orientados Pietra Graebin, Camilla Froes, Thiago Borges, aos mestres Diego Paskulin, Francis Paludo, Helena Strurow, Juliane Picanço e a minha Orientadora Clarice Alho pela dedicação a este nosso projeto.

LISTA DE ABREVIATURAS

APACHE II: Acute Physiology and Chronic Health Evaluation II

α-MSH: α-melanocyte-stimulating hormone – Hormônio estimulante de melanócito

bpm: batimentos por minuto

CAD: Coronary arterial disease

CD14: *Cluster of Differention* 14

m*CD14*: *CD14* de membrana

s*CD14*: *CD14* solúvel

cels/mm³: células por milímetro cúbico

CNPq: Conselho Nacional de Desenvolvimento Científico e Tecnológico

DNA: *Deoxyribonucleic Acid* – Ácido desoxirribonucléico

HIV: *Human lmunnedeficiency Vírus* – síndrome da imunodeficiência adquirida (SIDA)

HSP: Heat shock protein

ICU: Intensive Care Unit

IL: Interleucina

IAM: infarto agudo do Miocardio

IRAK: *IL-1R-associated kinase*

irpm: impulso respiratório por minuto

LBP: *Lopopolysaccharide Binding Proteins* – Proteína Ligante de Lipolissacarideo

LPS: Lipopolissacarídeos

LDL: lipoproteína de baixa densidade

mmHg: milímetros de Mercúrio

MODS: *Multiple Organ Dysfunction Syndrome* - Síndrome da disfunção de múltiplos órgãos

MyD88: *Myeloid Differentiation Factor* – Fator de diferenciação Mieloide

NCBI: *National Center of Biothecnology Information*

NF-kB: *Nuclear factor kB*- Fator Nuclear kB

PaCO2: pressão parcial de Dióxido de Carbono

PCR: Polymerase Chain Reaction – Reação em cadeia da polimerase

PCR RFLP: Restriction fragment length polymorphism - Polimorfismo do Tamanho do Fragmento de Restrição.

PRR: Pattern Recognition Receptor- Receptores de reconhecimento padrão

SIRS: Síndrome da Resposta Inflamatória Sistêmica

SNP: *Single Nucleotide Polymorphism*- Polimorfismo de um Único Nucleotídeo

SOFA: *Sequentical Organ Failure Assessment* – Avaliação seqüencial da falha de Órgãos

SPSS: *Statistical Package for the Social Sciences*

TGF-β: transforming growth factor β – Fatores transformadores de crescimento

TIR: *Toll Interleucine Receptor* - Receptor Toll Interleucina

TLR: *Toll-like Receptor* – Receptor Toll-like

TNF-α: Tumor Necrosis Factor alpha - Fator de Necrose Tumoral Alfa

TNFR1: *tumor necrosis factor receptor 1*

TNFR2: *tumor necrosis factor receptor 1*

TRAF6: *Tumor-necrosis-factor receptor-associated factor 6* – Receptor de fator de necrose tumoral associado ao fator 6

UTI: Unidade de Tratamento intensivo

UTIG-HSL-PUCRS: Unidade de Tratamento Intensivo Geral do Hospital São Lucas da Pontifícia Universidade Católica do Rio Grande do Sul

RESUMO

A condição crítica de saúde é causada pela interação de fatores genéticos e ambientais. Embora cada fator de risco em si já esteja parcialmente sob controle genético, estudos propõem a existência de efeitos adicionais causados por genes de susceptibilidade; estes estudos iniciaram sugerindo variantes genéticas isoladas que poderiam aumentar o risco do paciente criticamente enfermo.

Paralelamente, há evidências crescentes de que a inflamação desempenha também um papel central nos pacientes com condições críticas de saúde. Durante a situação crítica, os fatores de risco clínicos e bioquímicos convencionais são muito importantes, mas o estado inflamatório do paciente pode modular a gravidade do processo patológico. A inflamação pode estar envolvida em todas as fases do desenvolvimento e das conseqüências da doença crítica, sendo o processo inflamatório um agente central da morbi-mortandade do paciente criticamente doente. Assim, controlando o estado inflamatório pode-se aumentar a chance do indivíduo ter um melhor / pior desfecho.

O *CD14* (*cluster of diferenciation* 14) é um receptor padrão de reconhecimento de moléculas envolvidas na resposta imune inata contra fatores exógenos e endógenos de estresse. Os co-receptoes do *CD14* mais importantes são *TLR2*, *TLR4* (Toll-like Receptors), que são receptores transmembrana que mediam a resposta inflamatória por endotoxinas, e ativam a via do fator nuclear kappa B (NF-kappa B). O fator de necrose tumoral (*TNF-a*) é outra citocina relevante no âmbito do processo de inflamação. Mas, além de seu papel protetor na imunidade inata, essas citocinas pró-inflamatórias podem exercer também efeitos patogênicos.

Em 2006, foi avaliada a influência do polimorfismo de nucleotídeo único (SNP) -260C>T *CD14* em uma amostra de 85 pacientes criticamente enfermos. Com uma distribuição aleatória de genótipos para as características clínicas, como tempo de internação do paciente na Unidade de Terapia Intensiva (UTI), idade e tempo de permanência hospitalar, foi observado que os pacientes -260TT *CD14* apresentaram maiores índices de sobrevivência quando comparados com os portadores do alelo *-260C CD14*. Em 2009 foi testado uma amostra de 514 pacientes em estado crítico se o genótipo -260TT *CD14* ocorreria mais frequentemente entre os sobreviventes do que entre os pacientes falecidos. Este estudo publicado mostrou que os resultados de 2006 se confirmaram com uma maior robustez. O SNP -260C>T *CD14* foi um fator protetor para a sobrevivência em pacientes gravemente doentes: houve uma frequência superior de sobreviventes homozigotos -260TT *CD14*. Estes resultados surgiram com a hipótese de a maior frequência do genótipo -260TT *CD14* em pacientes de UTI sobreviventes seria, possivelmente, explicada por um efeito de sinalização na imunidade inata.

Naquele momento (2009), a literatura atual estava sugerindo que a análise de uma série de marcadores genéticos polimórficos poderia ser mais informativa do que a análise de um único polimorfismo. Ciente destas informações buscou-se a analisar SNPs em outros genes que codificam proteínas com ações sinérgicas com o *CD14* para verificar se eles também poderiam ser informativos no desfecho dos pacientes com condições críticas de saúde. Verificou-se a herança de variantes nos genes *TLR2, TLR4, e TNF-a*, os quais poderiam atuar em sinergia com o SNP *-260C>T CD14* durante a condição crítica. Foram obtidos resultados que mostraram que SNPs 2029C>T e 2258G>A do *TLR2*, 896A>G e 1196C>T do *TLR4* e o -308G>A do *TNF-a*, isoladamente, não desempenham um papel significantemente notável no desfecho da doença crítica. No entanto, ao se realizar uma análise combinada com a herança do -260C>T *CD14*, foi detectado uma taxa de sobrevivência significativamente maior no grupo de pacientes duplo homozigoto -260TT *CD14/*-308GG *TNF-a*. Na análise ajustada com o duplo genótipo as principais variáveis clínicas preditoras de mortalidade, foram observadas que o duplo genótipo -260TT *CD14/*-308GG *TNF-a* foi um fator importante de proteção para a sobrevivência. Conectado ao efeito benéfico do -260TT *CD14*, o genótipo -308GG *TNF-a* foi protetor contra a relatada superexpressão de *TNF-a* causada por alelo *-308A TNF-a*. Em conclusão, os resultados apóiam a hipótese de que a interação entre os SNPs funcionais -260TT *CD14* e -308GG *TNF-a* pode estar influenciando o desfecho de pacientes criticamente enfermos.

ABSTRACT

Critical condition is caused by interactions between genetic and environmental factors. Although each risk factor itself is partially under genetic control, studies propose the existence of additional effects caused by susceptibility genes; these studies begin suggesting isolated genetic variants that was increasing the risk of the critically ill patient. This proposition is now becoming evident.

In parallel, there is growing evidence that inflammation plays also a central role in patients with critical health conditions and in their outcome. During the critical condition, the conventional clinic and biochemical risk factors are very important, the inflammatory status can modulates the severity of the pathological process. Inflammation may be involved in all stages of critical ill development and compliances, and the inflammatory process a central agent of morbidity and mortality of critically ill patient. So, controlling inflammatory status may enhance individual chance of to acquire a better/worse outcome.

The *CD14* (cluster of differentiation 14) receptor is a pattern of recognition molecules involved in the innate immune response against exogenous and endogenous stress factors. The most important *CD14* signaling co receptors are the toll-like receptor 2 and 4 (*TLR2*, *TLR4*), which are transmembrane receptors that mediate inflammatory responses by endotoxins, and activate the nuclear factor kappaB (NF-kappaB) pathway. Tumor necrosis factor (*TNF-a*) is another relevant cytokine in the course of the inflammation process. But, besides its protector role in innate immunity, these pro-inflammatory cytokines may exert also pathogenic effects.

In 2006, it was evaluated the influence of the -260C>T *CD14* single nucleotide polymorphism (SNP) in a sample of 85 critically ill patients. With random genotype distribution for clinical characteristics at Intensive Care Unit (ICU) patient admission, age, and length of hospital stay, we found that -260TT *CD14* patients presented higher survivor rates when compared to the *-260C CD14* carriers. In 2009 it was tested in a sample of 514 critically ill subjects whether the -260TT *CD14* genotype would occur more commonly among ICU survivors than among decease patients. This published study showed that previous 2006 results were robustly confirmed. The -260C>T *CD14* SNP was a protective factor towards survival in critically ill patients; there was higher frequency of survivors in -260TT *CD14* homozygote. These results emerge with the hypothesis suggested that the higher -260TT *CD14* genotype frequency in ICU survivor patients was possibly explained by an effect on innate immunity signaling.

In that moment (2009), the current literature was suggesting that the analysis of a lot of polymorphic genetic markers could be more informative than the analysis of a single polymorphism. Aware of this information it was analyzed differential SNPs in other genes that encode *CD14* synergic proteins to examine if they could also be informative in patients with critical health conditions. We verified whether the shared inheritance of *TLR2*, *TLR4*, and *TNF-a* variants might act in synergy with *-260C>T CD14* SNP on the outcome from critical condition. The results for 2029C>T and 2258G>A of *TLR2*, 896A>G and 1196C>T of *TLR4* and the -308G>A of *TNF-a* SNPs alone did show a significantly remarkable role in the outcome not from critical illness. However, when performed a combined analysis with the *CD14* inheritance, it was detected a significant higher survivor rate in -260TT *CD14/*-308GG *TNF-a* double-homozygote group. In the adjusted analysis with double-genotype variable and the main clinical predictors to mortality, showing that the -260TT *CD14/*-308GG *TNF-a* double-genotype was a significant protective factor towards survival. Connected to the beneficial effect of -260TT *CD14*, the -308GG *TNF-a* genotype was protector against the reported overexpression of *TNF-a* caused by *-308A* rare allele. In conclusion, this results supported the hypothesis that the interaction between -260TT *CD14* and -308GG *TNF-a* functional SNPs may be influencing the outcome of critically ill patients.

SUMÁRIO

CAPÍTULO 1

1 FUNDAMENTAÇÃO TEÓRICA

1.1 Processo inflamatório

O processo inflamatório é uma resposta direcionada do organismo a um trauma endógeno ou a um agente infeccioso para, neste ultimo caso, evitar a proliferação microbiana da região afetada. Contudo, dependendo do grau, a inflamação pode ser muitas vezes mais danosa do que a ação causada pelo agente infeccioso [1].

Em um foco de inflamação, as células brancas são mobilizadas para a área agredida desencadeando o processo inflamatório, o qual é controlado e auto limitado. Quando ocorre lesão tecidual, seja causada por bactérias, trauma, agentes químicos, calor ou por qualquer outro fenômeno, ocorre a liberação de diversas substâncias que produzem alterações secundárias acentuadas nos tecidos lesionados, como: 1- Vasodilatação local e o consequente aumento excessivo do fluxo sanguíneo local; 2- Aumento da permeabilidade dos capilares com vazamento de grandes quantidades de líquido para o espaço intersticial; 3- Coagulação no espaço intersticial devido a grande presença de fibrinogênio e de outras proteínas que vazaram dos capilares e 4- Tumefação celular [1,2].

A magnitude da reposta inflamatória é crucial ao organismo. Assim como a insuficiência nesta resposta (ou imunodeficiência) pode levar à infecção generalizada, a resposta excessiva pode causar morbidade e mortalidade [3]. Se a inflamação se espalhar pela corrente sanguínea, como ocorre no choque séptico, sepse, meningite e trauma severo, a resposta inflamatória pode ser mais perigosa do que o estímulo que a gerou. O equilíbrio homeostático é restabelecido quando o processo inflamatório é diminuído pela ação de resposta dos agentes antiinflamatórios [3]. Esse equilíbrio da inflamação se dá por uma resposta altamente conservada de contra-resposta à inflamação, o processo antiinflamatório. Citocinas antiinflamatórias como interleucina-10 (IL-10) e *transforming growth factor β* (TGF-β – Fatores transformadores de crescimento), bloqueiam a ação do fator de necrose tumoral (TNF) e outros mediadores pró-inflamatórios, assim como glicocorticóides adrenais, adrenalina, *α-melanocyte-stimulating hormone* (α-MSH – Hormônio estimulante de melanócito) e outros hormônios de estresse clássicos inibem a produção de citocina e o sinal de transdução intracelular [3-5].

A inflamação está sempre presente nos locais que sofreram alguma forma de agressão e que, portanto, perderam sua homeostase. O processo inflamatório visa compensar essas alterações de forma e de função por intermédio de reações teciduais, principalmente vasculares, que buscam destruir o agente agressor. A inflamação pode ser considerada, assim, uma reação de defesa local [6]. O tempo de duração e a intensidade do agente inflamatório determinam diferentes graus ou fases de transformação nos tecidos, caracterizando uma inflamação como sendo, por exemplo, do tipo agudo ou crônico [6]. Classicamente, existem alguns fenômenos básicos comuns a qualquer tipo de inflamação e que independem do agente inflamatório. Esses momentos ou fases caracterizam a inflamação do tipo aguda, a qual

sempre antecede a inflamação do tipo crônica. Todos eles acontecem como um processo único e concomitante, o que caracteriza a inflamação como um processo dinâmico [6].

A manifestação clínica dessas fases se dá por intermédio de cinco sinais, intitulados sinais cardinais, que caracterizam a agudização do processo inflamatório. São eles: tumor, calor, rubor, dor e perda de função. O tumor é causado pelo aumento de líquido (edema inflamatório) e de células. O calor é oriundo da vasodilatação arterial e, consequentemente, aumento da temperatura local. O rubor ou vermelhidão também é decorrente desse mesmo fenômeno. A dor, por sua vez, é originada de mecanismos mais complexos que incluem compressão das fibras nervosas locais devido ao acúmulo de líquidos e de células, agressão direta às fibras nervosas e ações farmacológicas sobre as terminações nervosas. A perda de função, por fim, é decorrente do tumor (principalmente em articulações, impedindo a movimentação) e da própria dor, dificultando as atividades locais [6].

A inflamação aguda tem um curso rápido (entre 1 a 2 semanas), e a crônica constitui processos que superam três meses. A variação entre os processos agudos e crônicos está diretamente vinculada aos fatores que influenciam a inflamação [6]. De um modo geral diante de estímulos de grande intensidade no qual o hospedeiro consiga reagir e tornar de curta duração estes mesmos estímulos, torna-se presente o aparecimento de exsudação plasmático e neutrófilo, sinal característico de inflamação aguda [6]. Por outro lado, a persistência do estímulo - exigindo uma reação diferente da anterior por parte do hospedeiro - promove um aumento dos graus de celularidade (principalmente dos elementos mononucleares), o que determina uma fase proliferativa e reparativa e, portanto, de inflamação crônica [6]. Casos de curso agudo exibem intensa exsudação plasmática (migração, para o foco inflamatório, de líquidos e células, oriundos de vasos ou tecidos próximos), grande quantidade de neutrófilos e os sinais cardinais típicos da inflamação. A inflamação crônica perdura por longo tempo, não sendo visíveis os sinais cardinais (dor, tumor, calor, rubor e perda de função), presença de fibroblastos, linfócitos, macrófagos e pouca quantidade ou ausência exsudação plasmática [6].

Considera-se uma resposta inflamatória como sistêmica (síndrome da resposta inflamatória sistêmica ou SIRS) quando o paciente manifesta dois ou mais dos critérios a seguir: 1- Febre, temperatura corporal >38ºC ou hipotermia, temperatura corporal <36ºC; 2- Taquicardia, frequência cardíaca >90 bpm; 3- Taquipnéia, frequência respiratória >20 irpm ou $PaCO_2$ <32 mmHg; 4- Leucocitose ou leucopenia, Leucócitos >12.000 cels/mm^3 ou <4.000 cels/mm^3, ou presença de >10% de neutrófilos de formas jovens (bastões) [7]. A resposta inflamatória sistêmica está associada a um grande número de condições clínicas, além das infecções que podem produzir a SIRS. Causas patológicas não infecciosas podem incluir pancreatites, isquemia, traumas múltiplos e lesão nos tecidos, choque hemorrágico e administração exógena de alguns mediadores do processo inflamatório como o fator de necrose tumoral e outras citocinas. Uma frequente complicação da SIRS é o desenvolvimento de uma disfunção sistêmica dos órgãos, incluindo condições clínicas como lesão aguda nos pulmões, choque, falha renal e Síndrome da Disfunção Múltipla dos Órgãos (MODS; *Multiple Organ Dysfunction Syndrome*) [7].

O termo sepse é usado para os casos onde há SIRS mais um foco de infecção confirmado, sendo uma complexa desregulação da inflamação devida à incapacidade de se conter a infecção. Esta desregulação afeta múltiplos órgãos através de efeitos em células endoteliais, epiteliais e imunes que podem levar a um dano irreversível (Figura 1) [8].

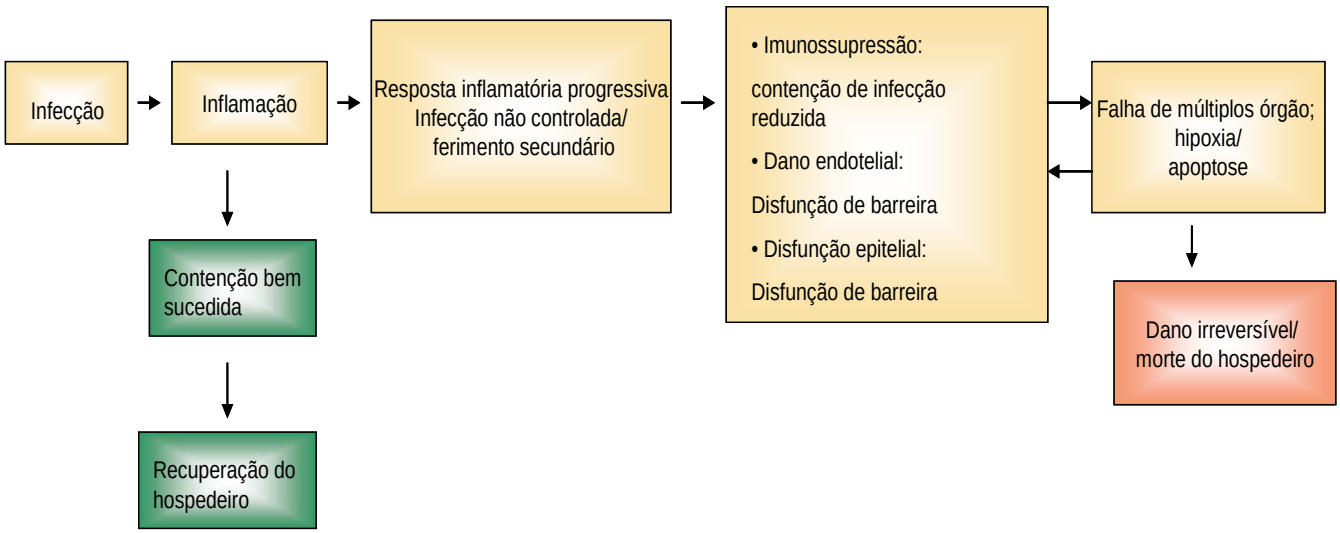

Figura 1: Patogênese da sepse, Adaptado de Buras *et al.,* 2005.

Choque é uma complicação caracterizada por hipotensão refratária, a qual é a principal causa do óbito decorrente de SIRS. Se a hipotensão ou hipoperfusão são refratárias à ressuscitação volêmica, e se a administração de agentes vasopressores não oferece resposta adequada, as complicações circulatórias podem levar à falência ou à disfunção de múltiplos órgãos (MODS; *Multiple Organ Disfunction Sindrome*). A falência de múltiplos órgãos é uma alteração tão severa na função orgânica que sua homeostasia não pode ser mantida sem intervenção terapêutica artificial [9]. O choque decorrente da sepse (SIRS+foco de infecção) pode ter o mesmo desfecho (figura 2).

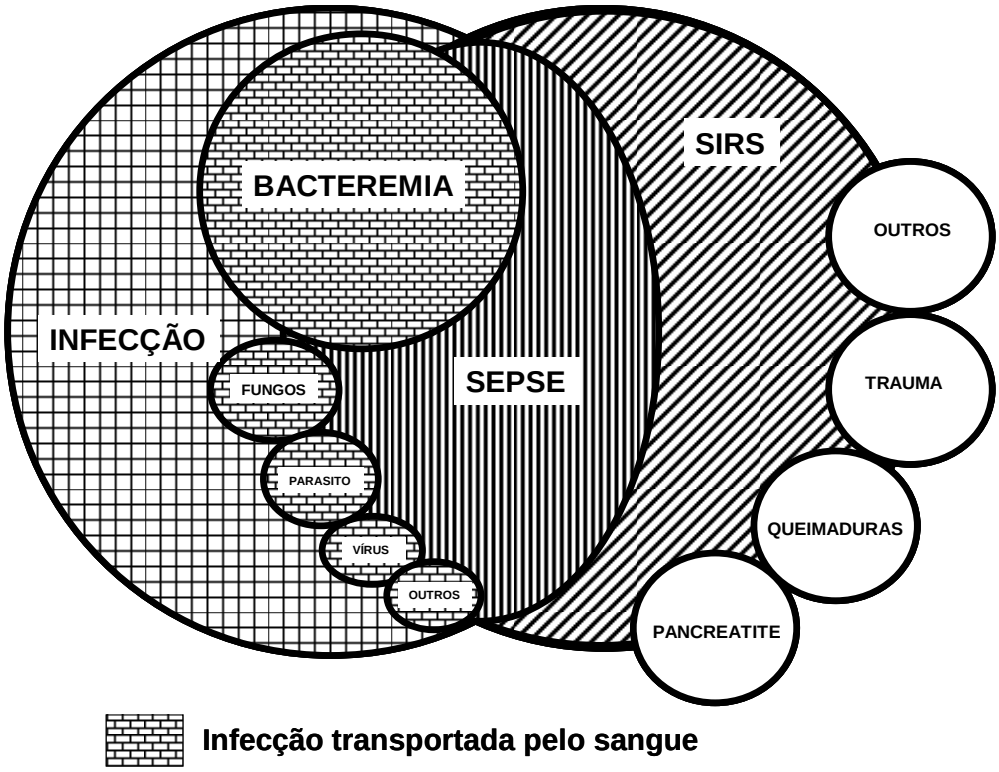

Figura 2: Inter-relação entre SIRS, sepse e infecção. Adaptado de Bone *et al.,* 1992.

No quadro de choque a vasodilatação periférica é evidenciada e há excessiva presença de agentes pró-inflamatórios que, juntos, acentuam a disfunção e a falência de órgãos. Detectam-se evidências de disfunção muito antes da falência de órgãos, resultado da reação inflamatória pela massiva liberação de citocinas. A liberação concomitante de agentes pró-inflamatórios e antiinflamatórios será o que manterá a homeostasia do organismo, podendo haver uma reação antiinflamatória maior e, algumas vezes, mais longa que a pró-inflamatória a fim de conservar o equilíbrio homeostático [7, 10]. Se a reação pró-inflamatória não for equilibrada pode haver o desencadeamento de um processo inflamatório crônico letal.

A inapropriada ativação do sistema imune desempenhará um papel fundamental na propagação de um quadro de enfermidade. Entre os fatores desencadeantes da resposta imune estão os antígenos endógenos, como a LDL (lipoproteína de baixa densidade) oxidada ou as proteínas de choque térmico (*heat shock proteins -HSP*), e os antígenos exógenos patogênicos, tais como as proteínas de choque térmico exógenas ou o lipopolissacarídeo bacteriano (LPS), que se associam aos padrões moleculares (receptores de reconhecimento padrão; *patern recognitions receptor*) [11].

1.2 Imunidade Inata

Ao contrário da imunidade adaptativa, a qual é específica e se molda ao agente infeccioso criando uma memória imunológica, o sistema imune inato reconhece classes genéricas de moléculas endógenas e as produzidas por vários microorganismos patogênicos. As moléculas envolvidas na imunidade inata são as primeiras linhas de defesa do organismo, desempenhando um papel fundamental nas doenças inflamatórias e infecciosas, uma vez que desencadeiam uma resposta inflamatória generalista, na qual certas células (macrófagos, monócitos, granulócitos e células dendríticas) detêm o agente invasor, impedindo que ele se espalhe [12]. Uma função importante para a resposta imune inata é de recrutar mais células fagocitárias e moléculas efetoras para o local da inflamação/infecção, através da liberação de uma bateria de citocinas e de outros mediadores inflamatórios que têm profundos efeitos sobre os fatos subseqüentes. As citocinas pró-inflamátorias, cuja síntese é estimulada quando os macrófagos iniciam a resposta ao processo inflamatório/infeccioso, compreendem um grupo estruturalmente diferenciado de moléculas e incluem as interleucinas IL-1, IL-6, IL-8, IL-12 e o fator de necrose tumoral (*TNF-α*), todos apresentam importantes efeitos locais e sistêmicos [11, 13]. Os efeitos combinados desses mediadores contribuem para as reações locais contra a infecção na forma de resposta inflamatória [14, 15]. A resposta inflamatória operacional, já anteriormente citada, é caracterizada pela dor, rubor, calor e pelo tumor no sítio da inflamação [16]. O tumor gera alterações que consiste num aumento do diâmetro vascular, levando a um aumento do volume sanguíneo local [17]. Uma vez que a inflamação/infecção se dissemine para a corrente circulatória, os mesmos mecanismos através dos quais o *TNF-α* continha a inflamação/infecção local com tanta eficiência, tornam-se ineficazes [2, 18].

O processo da imunidade inata, de resposta rápida para detectar e eliminar a inflamação/infecção é iniciado pela molécula *CD14* (*cluster of differenciation* 14) e mediado pelos receptores Toll-Like (*Toll-like receptors*; TLRs), os quais fazem parte de uma família de receptores conservada desde artrópodes até mamíferos [19-21]. Antígenos endógenos e/ou exógenos ligam-se ao *CD14* e provocam uma sinalização transmembrana através do *TLR2* ou do *TLR4* [22]. O *CD14*, o *TLR2* e o *TLR4* estão, portanto entre as principais proteínas envolvidas nas condições inflamatórias.

1.3 O CD14

O *CD14*, uma glicoproteína de 55 KDa, pode ser encontrado fixo na membrana de células do sistema imune (m*CD14*) ou livre no soro (s*CD14*). O *CD14*, contudo, não tem conexão com o citoplasma celular não podendo ativar a resposta imune isoladamente [23]. A mediação do processo imunológico inato se dá pelos TLRs [24,25]. O reconhecimento de antígenos endógenos/exógenos pelo sistema imune inato desencadeia a resposta inflamatória com o objetivo de acabar com o foco inflamatório. Uma resposta inflamatória exacerbada e sem controle pode, contudo, ser prejudicial, sendo a causa de disfunções orgânicas importantes [12].

O *CD14* é expresso na superfície (m*CD14*) de células mielóides (monócitos, macrófagos e neutrófilos), bem como de células não-mielóides (endotelial e epitelial) [26]. O *CD14* solúvel (s*CD14*), que aparentemente decorre da secreção direta de *CD14* ao meio extracelular ou da proteólise do m*CD14* [27,28], desempenha um papel crucial na função imune das células que não expressam m*CD14* por que lhes permite desencadear a síntese de citocinas inflamatórias [29-31].

Dentre os polimorfismos encontrados para o gene *CD14* com relação à mensuração dos níveis de *CD14* [rs2569191 (-1145G>A), rs5744455 (-550C>T ou -651C>T), rs60313457 (-159C>T ou -260C>T) e rs4919 (6819C>G)] escolhemos o rs60313457 (-159C>T ou -260C>T) que é o mais bem estudado e relacionado a expressão do *CD14* em relação a processos inflamatórios e infecção. Este *single nucleotide polymorphism* (SNP) foi identificado na sequência do promotor do gene *CD14*, localizado no locus 5q23-31, uma transição de citosina (C) a timina (T) na posição -260 do sitio inicial de tradução do gene (-260C> T; rs60313457) (figura 3) [32]. Esse polimorfismo é encontrado perto do local de reconhecimento para o fator de transcrição Sp1 e parece ter um papel importante na regulação *CD14* [33, 34], uma vez que pode interferir de forma quantitativa a capacidade de transcrição do gene *CD14*. Em 2006, foi avaliada a influência do -260C>T SNP do *CD14*, em uma amostra de 85 pacientes criticamente doentes do sul Brasil [35]. Mesmo com essa pequena amostra, as freqüências genotípicas e alélicas foram similares a outros estudos, com cerca de mil ou mais indivíduos de populações diferentes com os mesmos componentes de raízes étnicas européias [36-38], e estiveram em equilíbrio de Hardy-Weinberg. Em 2008 uma pesquisa utilizando 14 pacientes diagnosticados nos estágios iniciais da sepse comparados com voluntários saudáveis mostrou que homozigotos -260TT apresentaram maiores níveis de m*CD14* ($p=0.0027$), assim como a densidade de monócitos de m*CD14* e s*CD14* também foi encontrada elevada em pacientes sépticos em relação ao grupo controle ($p<0.001$) [39]. Estudos demonstraram que alelo -260C foi associado com risco aumentado de severa sepse (228 indivíduos) [40] e mortalidade (233 indivíduos [41]). Em contraste, alguns estudos não revelaram associação entre o *SNP -260C> T CD14* e sepse (204 pacientes e 247 controle, 58 indivíduos) [42,43] ou doenças infecciosas (77 e 39 pacientes de controle; 134 indivíduos) [24,44].

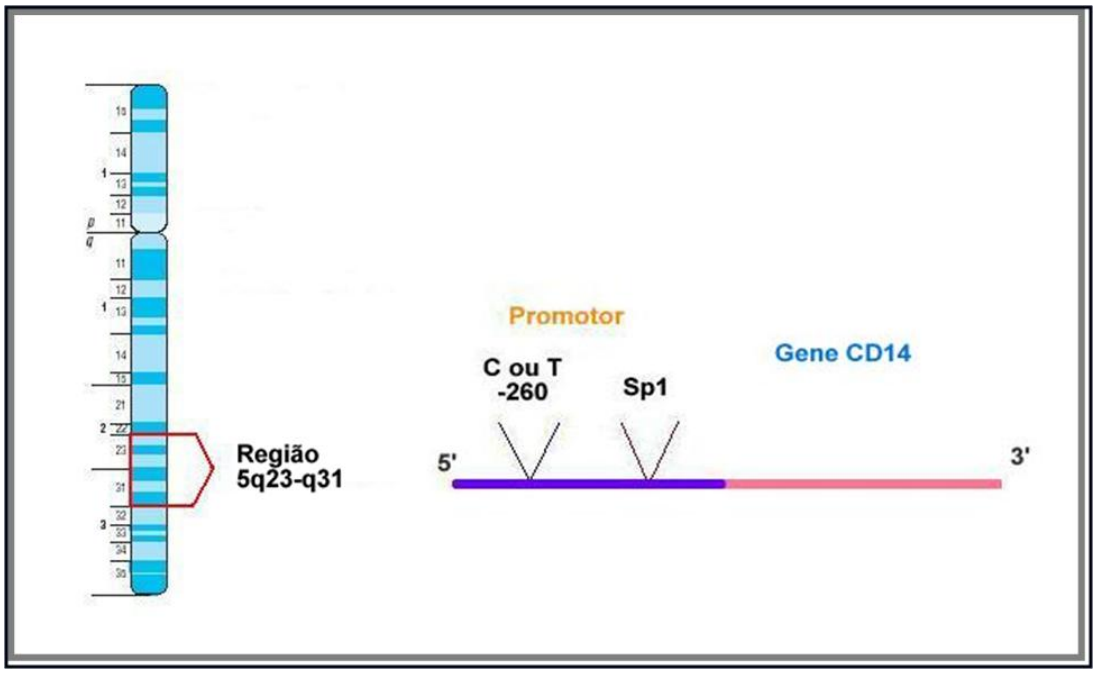

Figura 3: Região no Cromossomo 5 onde se encontra o SNP -260C>T do *CD14*.

1.4 Receptores Toll-like (TLRs)

Os TLRs são proteínas transmembrânicas que, em vertebrados, servem como estimuladores da interação do sistema inato com o adquirido. São também conhecidos como Receptores de Reconhecimento Padrão (*pattern recognition receptor*; PRR). Seu domínio extracelular consiste em um número variado de repetições ricas em leucina (Leu) e regiões ricas em cisteina (Cys) precedentes ao domínio transmembrana. O domínio citoplasmático é chamado de receptor Toll-interleucina-1 (*toll interleucine receptor*; TIR) [45-47].

A principal função dos TLRs, associada ao controle da resposta inflamatória e resposta imune, foi bem demonstrada na sua análise em camundongos com TLRs *knockout*. O nome Toll é derivado de uma sequência homologa da *Drosophila spp*, o gene *Toll* [48], o qual foi descoberto em 1996 e é relacionada com a formação do eixo ventral dorsal, assim como, com a resposta imune a infecção fungal [48]. A identificação entre a similaridade do domínio citoplasmático do Toll de *Drosophila spp* e o receptor IL-1 de mamíferos impeliram a busca por receptores ortólogos, subsequentemente levando a descoberta do primeiro Toll humano [49]. Estima-se que haja mais de dez diferentes tipos de TLRs, em humanos (figura 4) [48, 50,51].

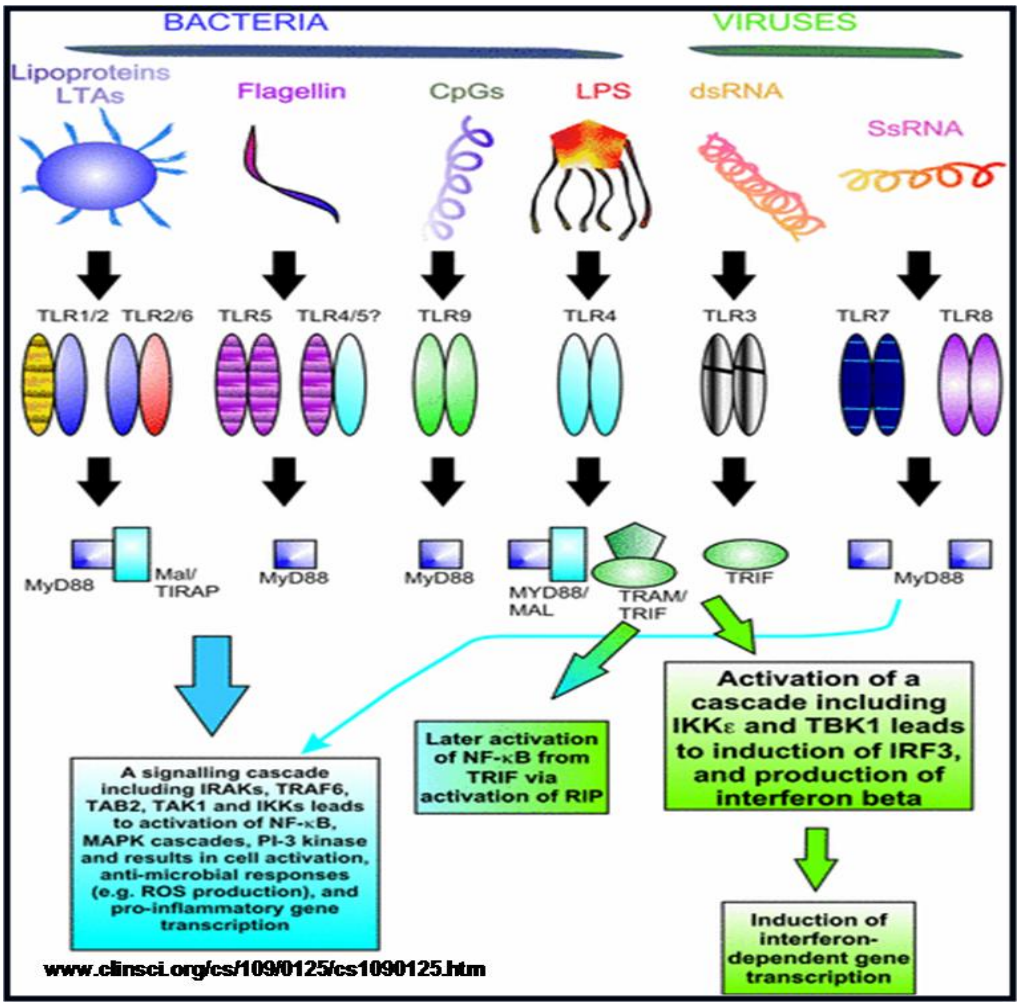

Figura 4: Representação dos TLRs e os tipos de componentes responsáveis por desencadear a sinalização para produção de citocinas pró-inflamatorias e suas cascatas.

Toll-like-Receptor 2 (TLR2)

O Toll-like Receptor 2 (*TLR2*) reconhece componentes de uma variedade de antígenos, especialmente de microorganismos. Entretanto, o *TLR2* liga-se aos antígenos com baixa afinidade [52], requerendo a ação conjunta com o *CD14* [53]. O gene que codifica para o *TLR2* foi mapeado no cromossomo 4 (figura 5), no *locus* 4q31.3-32 [54,55].

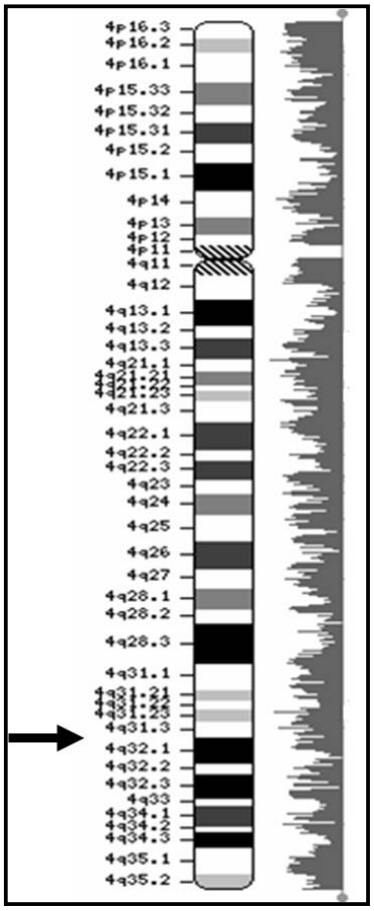

Figura 5: Mapa do cromossomo 4 que contém o *locus* gênico para o *TLR2*. A seta indica o *lócus* estudado. [NCBI: Map Viewer, em http://www.ncbi.nlm.nih.gov,acessado em 28 de setembro de 2007].

Texereau *et al*. indicaram as principais alterações polimórficas descritas no gene do *TLR2* (Figura 6) [56] e Merx *et al* também caracterizaram alguns polimorfismos importantes (Figura 7) [57].

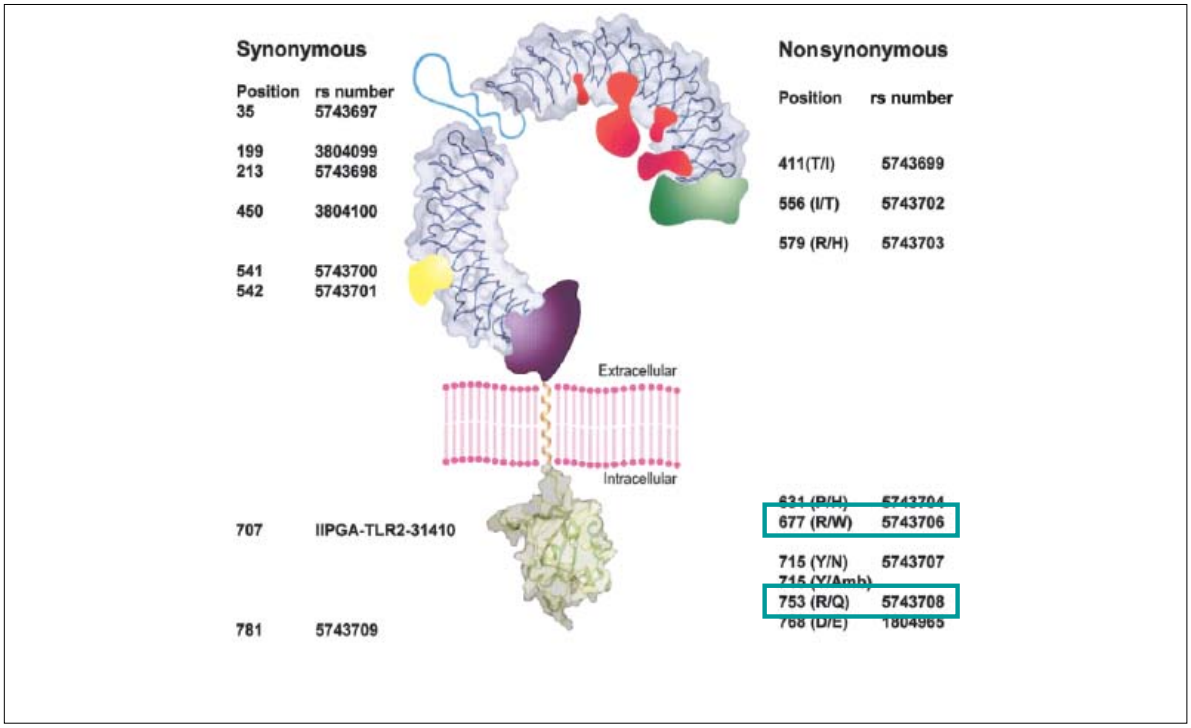

Figura 6: Principais variantes polimórficas do gene do *TLR2* ilustradas por Texereau *et al*, 2005. Em destaque nos quadros estão os SNPs estudados neste trabalho.

Database results				
cDNA (nt position)	gDNA (nt position)	refSNP_ID	SNP (amino acid position)	Heterozygosity frequency
597T>C	817T>C	rs3804099	Asn199Asn	0.486
639G>C	859G>C	rs5743698	Leu213Leu	0.021
1232C>T	1452C>T	rs5743699	Thr411Ile	0.008
1350T>C	1570T>C	rs3804100	Ser450Ser	0.239
1623C>T	1843C>T	rs5743700	Phe541Phe	0.044
1626C>G	1846C>G	rs5743701	Leu542Leu	0.044
1667T>C	1887T>C	rs5743702	Ile556Thr	n.a.
1736G>A	1956G>A	rs5743703	Arg579His	0.008
1892C>A	2112C>A	rs5743704	Pro631His	0.035
2121T>C	2341T>C	rs5743705	Phe707Phe	n.a.
2143T>G	2341T>G	rs5743707	Tyr715stop	0.004
2143/45TAT>AAA	2363/65 TAT>AAA	n.a.	Tyr715Lys	CHIP + innate immunity
2258G>A	2478G>A	rs5743708	Arg753Gln	0.028
2304G>T	2524G>T	rs1804965	Glu768Asp	n.a.
1339C>T	1559C>T		Arg447stop	n.a.

SNP information about human TLR2 obtained from human genetic database research. cDNA, coding DNA; gDNA, genomic DNA; het.frequency, heterozygosity frequency; Het, heterozygote; Hom, homozygote; n.a., not available.

Figura 7: Caracterização dos principais polimorfismos do *TLR2* segundo Merx *et al*, 2007. Em destaque no quadro está um dos SNPs estudados neste trabalho.

Dentre todos os polimorfismos relacionados ao gene que codifica para o *TLR2*, somente dois deles têm sido mais estudados nos últimos anos e têm sido supostamente relacionados à redução da ativação do NF-kB e ao aumento do risco de infecção, confirmando modelos animais que sugerem que uma sinalização defeituosa do *TLR2* é um fator causal para o aumento na susceptibilidade a doenças bacterianas [56]. O primeiro SNP consiste em uma substituição de uma citosina (C) por uma timina (T) no nucleotídeo 2029 (2029C>T) que resulta na modificação de uma Arginina em um Triptofano no aminoácido 677 (Arg677Trp) do gene humano do *TLR2* e foi primeiramente relacionado com pacientes com Hanseníase [58]. Posteriormente foi também relacionado com a suscetibilidade à Tuberculose [59].

O segundo SNP descrito como a substituição de uma guanina (G) para uma adenina (A) no aminoácido 2258 (2258G>A) gerando a transição de uma Arginina por uma Glutamina no resíduo 753 (Arg753Gln) da proteína do *TLR2*. Este polimorfismo foi primeiramente descrito por Lorenz *et al*. [60], que identificaram que esta mutação ocorria em 3% da população testada. O estudo afirma que, *in vitro*, o SNP 2258G>A não afeta a habilidade do *TLR2* em responder ao LPS, mas afeta na habilidade do *TLR2* em responder a peptídeos bacterianos. Além disso, foi realizado um estudo de caso-controle para verificar se a ocorrência da mutação em pacientes com choque séptico infectados com bactérias Gram-positivas era mais frequente do que em pacientes infectados por bactérias Gram-negativas. Foram encontrados, em 91 casos e 73 controles estudados, 22 pacientes com choque séptico causado por bactérias Gram-positivas. Destes, dois pacientes carregavam a mutação 2258G>A e também apresentavam infecção por estafilococos. Assim, foi sugerido que o alelo mutante 2258A poderia ser um fator de risco para o desenvolvimento de choque séptico após a infecção por bactérias Gram-positivas [60].

Schröder *et al*. [61] publicaram que o alelo 2258A ocorria em 9,4% da população estudada (europeus), o que era uma porcentagem significativamente maior do que a encontrada anteriormente por Lorenz *et al.* [60]. Os autores ainda salientaram que o fato de o SNP 2258G>A estar presente em uma maior frequência em europeus faz deste um alvo para estudos que correlacionem este polimorfismo com a incidência de doenças infecciosas/inflamatórias [61].

Posteriormente, Hamann *et al.* [62], relataram um aumento significativo na frequencia do alelo 2258A entre pacientes que desenvolveram reestenose, em comparação aos que não desenvolveram, suportando, assim, um papel do *TLR2* de induzir inflamação na doença cardiovascular. Ainda, Schröder *et al*. [63], indicaram que o alelo 2258A do *TLR2* poderia proteger do desenvolvimento do estado tardio da Doença de Lyme e foi verificado que o SNP 2258G>A contribuía significativamente para a patogênese da Febre Reumática Aguda em crianças [64]. Em um estudo mais recente, Thurow *et al.* mostraram que a presença dos alelos raros para os SNPs 2258G>A e 2029C>T do *TLR2* na população do sul do Brasil era insignificante [65].

Toll-like receptor 4 (TLR4)

O *TLR4* é responsável pela mediação do reconhecimento aos antígenos endógenos e exógenos e pela sinalização para liberação das citocinas. O receptor *CD14*, o qual não possui porção intracelular [23], aciona o *TLR4* através da molécula adaptadora MD2 [48]. A transdução do sinal tem início pela interação do domínio TIR do *TLR4* com outro domínio TIR presente na molécula citoplasmática denominada MyD88 (*Myeloid differentiation protein* 88) [64]. O MyD88 possui um caminho independente envolvendo proteínas com domínios TIR. O *death-domain* do MyD88 se prende ao *death-domain* de uma serina/treonina kinase (em geral, uma kinase IRAK-Interleukin-*1-receptor-associated kinase*) e se propaga via TRAF6 (*TNF-receptor-associated factor 6*) [66]. TRAF6 induz a produção de um regulador mestre de inflamação, o NF-κB [67]. Esse regulador aciona os genes que codificam os ativadores imunes, o que inclui o *TNF-α* e as interleucinas IL-1, IL-8, IL-12 e IL-6 [45] (figura 8).

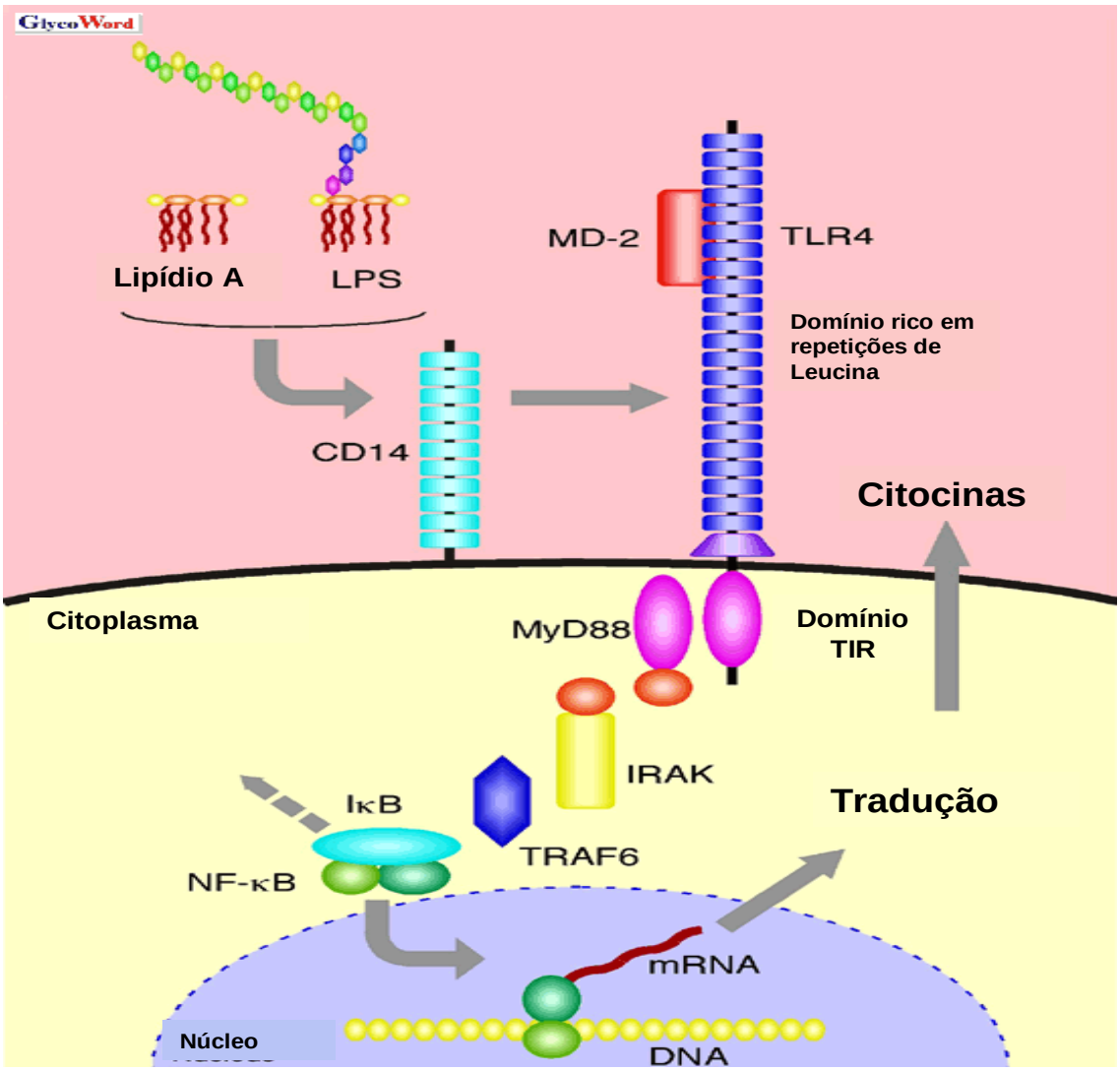

Figura 8: Mecanismo de desencadeamento do processo da resposta imune inata provocado por LPS de bactérias Gram-negativas nas seguintes ligações e ativações: LPS→LPB→*CD14*→MD2→*TLR4*→NF-κB→citocinas. Extraído do site http://www.glycoforum.gr.jp/science/word/immunity/IS-A01J.html

Diversos SNPs do *TLR4* são alvo de estudos relacionados a doenças inflamatórias e a hiporesponsividade da resposta imune do individuo, entre esses SNPs podemos citar algumas referencias [1859 G>A (rs11536858), 2437A>G (rs1927914), 7764G>A (rs1927911), rs12377632, 11911 G>T (rs2149356), 3725G>C rs11536889, 2032T>C (rs10116253), 11912 G>T (rs2149356), 896A>G rs4986790 e 1196C>T rs4986791)] [68-70]. Os dois últimos SNPs relacionados (896A>G rs4986790 e 1196C>T rs4986791) do *TLR4* despontam nas pesquisas publicadas referentes a processos inflamatórios e com hiporresponsividade a processos infecciosos. O primeiro SNP foi mapeado no cromossomo 9 (*locus* 9q32-q33) [68-72] e nele foram descritas duas alterações polimórficas que alteram a estrutura protéica do *TLR4* humano: 1- uma substituição de um único nucleotídeo (SNP), dentro do éxon 4 do gene *TLR4*, de uma adenina (A) para uma guanina (G) na base 896 (896A>G), que leva à modificação do resíduo conservado de ácido aspártico para uma glicina no aminoácido 299 da sequência protéica (Asp299Gly) do domínio da estrutura extracelular do *TLR4* [71,73]; e 2- uma transição de uma citosina (C) para uma timina (T) na posição 1196 (1196C>T) que gera a substituição do aminoácido treonina por uma isoleucina no resíduo 399 (Thr399Ile) da região extracelular do *TLR4* [24,71,74]. A incidência da maioria dos polimorfismos nos seres humanos é extremamente baixos (< 1%), com duas exceções (896A>G e 1196C>T) ambas variantes nas populações apresentam uma freqüências alélicas de *896G* e *1196T*, variando de 6 a 10%) e parecem promover dificuldades no reconhecimento do *TLR4* ao LPS [24,68,71,73,74].

As variantes polimórficas mutantes *896G* e *1196T* foram encontradas em proporções significativamente mais elevadas em pacientes com uma baixa resposta imune (indivíduos hipo-responsivos) (6.6%) quando comparados à população controle [63,70]. Células epiteliais derivadas destes pacientes exibiram uma diminuição na resposta imune no estado homozigótico (896GG e 1196TT) ou heterozigótico (896AG e 1196CT) [66]. Achados similares foram observados em estudos *in vitro* pela análise do soro e medição de níveis das citocinas em macrófagos alveolares (9.6%) [75]. Estes resultados levaram à sugestão de que as variantes mutantes 896G e 1196T diminuiriam a função de sinalização da imunidade inata do *TLR4*.

O que se torna muito intrigante ao se realizar um estudo meta-analítico destas variantes polimórficas que afetam a ação do *TLR4* é que, embora humanos com as variantes 896G e/ou 1196T do gene *TLR4* apresentem-se mais resistentes a uma resposta inflamatória induzida (indivíduos hiporesponsivos), em algumas populações, esses indivíduos estão mais suscetíveis a uma resposta inflamatória sistêmica [76]. Assim, foi observado que a herança das variantes 896G e/ou 1196T está relacionada à propensão a ocorrência de diabetes I e II, de arteriosclerose [77], infarto agudo do miocárdio (IAM) e doença arterial coronariana (CAD)[75]. Foi encontrada uma associação entre a baixa resposta imune (indivíduos hiporesponsivos) relacionada à herança das variantes 896G e/ou 1196T e o risco a diabetes e a IAM, mostrando a prevalência de pacientes portadores das variantes 896G e 1196T do gene *TLR4* com IAM [75].

Foi observada uma associação entre a herança do alelo 896G e níveis reduzidos de proteína C reativa (CRP), a qual é um marcador de resposta às inflamações. No entanto, no referido estudo, investigando pacientes sem IAM que passaram por uma angiografia, os autores observaram que portadores do alelo 896G tinham reduzida prevalência de CAD e diabetes I e II. Este achado sugeriu que uma menor resposta a regulação do sistema imune pode ser benéfica, modificando o risco de CAD e da diabete [77].

1.5 O TNF-Alpha

Usualmente chamado de fator de necrose tumoral, o TNF pode também ser encontrado na literatura como TNFA, *TNF-α*, TNF derivado de monócitos e TNF derivado de macrófagos. O *TNF-α* é uma citocina pro - inflamatória multifuncional, secretada predominantemente por monócitos e macrófagos, que possui efeitos no metabolismo de lipídeos, na coagulação sanguínea, na resistência à insulina e na função endotelial [78]. *TNF-α* foi primeiramente identificado em soro de camundongos após administração de toxina bacteriana, tornando-o citotóxico ou citostático para células humanas levando-as à necrose hemorrágica [79,80]. *TNF-α* é sintetizado como uma proteína de membrana de 26kDa, e ao ser clivado por uma metaloproteinase [81], torna-se solúvel em sua forma ativa com 17kDa. As moléculas de *TNF-α* com 17kDa formam trímeros que se ligam aos dois receptores de membrana de *TNF-α*: o receptor do fator de necrose tumoral 1 (TNFR1 – *tumor necrosis factor receptor 1*) e o receptor do fator de necrose tumoral 2 (TNFR2 – *tumor necrosis factor receptor 2*) [82], sendo que a maioria dos efeitos biológicos do *TNF-α* está relacionada ao TNFR1. A expressão de *TNF-α* é regulada de diferentes formas, tanto durante a transcrição como na tradução, e através da análise da sequência promotora do gene foi encontrada uma série de sítios de ligação de fatores de transcrição que apresentam papel importante na transcrição do gene [83], como por exemplo, NF-KB, AP-1, AP-2, CREB, Egr-1, e Sp1 [84]. A regulação do processo de transcrição do gene *TNF-α* é essencial para evitar os efeitos deletérios de síntese inapropriada ou excessiva de *TNF-α* [85]. A expressão inapropriada ou superexpressão de *TNF-α* podem levar à progressão da inflamação e às doenças auto-imunes [86-88].

O gene que codifica o *TNF-α* está localizado no braço curto do cromossomo 6, na região 6p21.3, está localizado na região principal do complexo de histocompatibilidade (MHC), e um grande número de polimorfismos do promotor foram descritos [88-91]. O gene do *TNF-α* é altamente polimórfico e é comumente relacionado a doenças infecciosas [90]. Uma série de polimorfismos já foi identificada na região promotora do gene, incluindo um SNP na posição -308 em relação sítio do início da transcrição, onde a presença de uma guanina (G) define o alelo comum, chamado de TNF1, e a presença de uma adenina (A) define o alelo mutante, chamado de TNF2 [92,93]. Wilson *et al.* demonstraram que o alelo selvagem é um potente ativador transcricional em relação ao alelo nativo, pois a presença do alelo "A" aumentou de seis a sete vezes o poder de transcrição de *TNF-α* [94]. Kroeger *et al.* também demonstraram que o SNP na posição -308 afeta o processo de transcrição [95]. A região localizada entre os nucleotídeos -323 e -285 (incluindo o SNP -308G>A) na presença do alelo "G" liga-se a quatro

complexos protéicos promotores da transcrição (B, C, D, e DI). Já na presença do alelo "A", ocorre a ligação de um quinto complexo proteico, denominado complexo E, que possui efeito na capacidade de ligação dos complexos proteicos comuns (B, C, D, e DI), aumentando a atividade transcricional do gene [95]. Wu *et al.* também demonstraram que o SNP -308G>A afeta a ligação de fatores de transcrição na região -347 a -269 do gene do *TNF-α*, aumentando de três a sete vezes a produção de *TNF-α* [96].

A produção excessiva de citocinas proinflamatórias não apenas aumenta o poder da resposta imune frente a um organismo invasor, como também pode apresentar efeitos deletérios que modificam a regulação hemodinâmica e o controle metabólico [97]. Embora um processo infeccioso seja complexo, a influência da herança genética do *TNF-α* (citocina proinflamatória produzida em altos níveis durante esse processo) tem sido examinada em relação à sepse, ao choque séptico e à mortalidade [85, 93, 98, 99, 100-104]. Mira *et al.* observaram que ser portador do alelo "A" pode estar associado a um risco maior de desenvolvimento de choque e mortalidade (n=89), a qual foi de 52% em comparação a 24% no grupo controle (P=0,008) [99]. Na sepse severa, os não sobreviventes apresentam uma prevalência significativa do alelo "A". Os homozigotos para o alelo "A" apresentam uma taxa de mortalidade elevada em relação aos heterozigotos (P=0,0022), além de concentrações mais elevadas de *TNF-α* e de um grau maior de disfunções orgânicas [105]. Estes achados foram corroborados em pacientes com trauma e submetidos à cirurgia [90], mas não em pacientes com pneumonia (n=280) [106].

Um estudo realizado em Taiwan, não mostrou diferença na freqüência alélica entre sobreviventes e não sobreviventes em relação à herança da variação polimórfica -308G>A em pacientes com choque séptico [107]. No subgrupo de pacientes com choque séptico (n=42), os autores encontraram que uma proporção significativamente maior de não sobreviventes possuía pelo menos uma cópia da variante alélica "A" em comparação com os sobreviventes. A mortalidade nos pacientes com pelo menos uma variante alélica "A" foi de 92% em comparação com 62% ($p<0,05$) nos pacientes homozigotos GG. Os autores também relataram níveis de *TNF-α* mais elevados nos pacientes com choque séptico que não sobreviveram, em comparação com os sobreviventes ($p<0,05$) [107]. O desfecho no choque séptico associou-se com o SNP -308G>A em pelo menos dois estudos [105,107]. Em relação a estes resultados, Stüber não considera que a genotipagem deste SNP em pacientes com sepse severa contribua para a determinação do risco. Em pacientes com sepse abdominal, os níveis de *TNF-α* não foram influenciados por este SNP [107].

1.6 Pacientes internados em Unidade de Terapia Intensiva

Pacientes internados em Unidades de Terapia Intensiva (UTIs) apresentam quadro patológico crítico e complexo decorrente de fragilidades fisiológicas graves, que são responsáveis pela elevada taxa de mortalidade que varia de 20% a 50% [108-111].

Nos últimos 20 anos, instrumentos de medida de predição de risco têm sido aplicados aos pacientes críticos internados em UTIs na tentativa de adoção das melhores estratégias terapêuticas possíveis. A avaliação do quadro clínico de pacientes internados em UTIs é realizada principalmente através de instrumentos que analisam a disfunção de órgãos e sistemas por meio do monitoramento diário de seus estados fisiológicos, porém, nenhum destes instrumentos avalia as predisposições genéticas de pacientes. O escore APACHE II (*Acute Physiology and Chronic Health Evaluation II*) considera, no dia da internação, 12 variáveis fisiológicas (levando em conta os piores valores das primeiras 24 horas da admissão), a presença de doença crônica e a idade, gerando um escore que avalia a severidade do estado patológico do paciente [112]. Ao contrário do escore APACHE II – que é gerado apenas no dia da internação – o escore SOFA (*Sequential Organ Failure Assessment*) avalia diariamente a condição de seis sistemas orgânicos (respiratório, renal, hepático, hematopoiético, cardiovascular e neurológico), independentemente de qualquer terapia a qual o paciente esteja sendo submetido [113].

Os pacientes internados em UTIs são indivíduos afetados por múltiplas disfunções orgânicas e que, além disto, estão expostos ao ambiente hospitalar, rico em diversidade de microorganismos infecciosos. Estudos epidemiológicos demonstram que aproximadamente 2% de todos os pacientes hospitalizados e 75% dos pacientes internados em UTIs acabam desenvolvendo processos inflamatórios exacerbados, com taxa de mortalidade entre 20-50%. Esses estudos demonstram que ocorrem aproximadamente 750.000 casos/ano nos EUA, com aumento estimado em 1,5%/ano [108-113]. Analisando 884 pacientes brasileiros que permaneceram pelo menos um dia internados na UTI, a incidência e a mortalidade decorrente de processos inflamatórios exacerbados foram, respectivamente, 47% e 35% [23].

Considerando a importância social da doença, principalmente no que concerne à taxa de morbi-mortalidade e prevalência, faz-se necessário intervenções de natureza preventiva e curativa. Várias iniciativas têm sido propostas no sentido de prevenir as infecções hospitalares, diminuindo esta parcela significativa de pacientes com alto risco de óbito. Do ponto de vista terapêutico, o insucesso tem sido a rotina em dezenas de estudos clínicos que almejaram controlar a resposta inflamatória sistêmica [114], tanto que a taxa de mortalidade não tem se alterado nos últimos anos [115].

O estudo da resposta inflamatória deve contribuir para levantamentos epidemiológicos, direcionados para o conhecimento dos mecanismos moleculares e celulares que desencadeiam as variações fisiopatológicas. Esse conhecimento básico poderá contribuir para a modulação da sequência de eventos que culminam nos desfechos desfavoráveis do paciente crítico [116].

2. JUSTIFICATIVA, HIPÓTESE

A condição crítica de um paciente, a gravidade de suas disfunções orgânicas e a evolução ou não para o óbito são determinadas pela interferência simultânea de inúmeros fatores externos e de fatores herdados. O estudo dos SNPs -260C>T do gene que codifica o *CD14*, 2258G>A e 2029C>T do gene que codifica o *TLR2*, 896A>G e 1196C>T do gene que codifica o *TLR4* e -308G>A do gene que codifica para o *TNF-α* pode ser útil para a identificação dos efeitos que as variantes genéticas podem ter no momento da condição crítica de saúde.

Compilando os resultados de diferentes estudos foi possível sugerir que pacientes que apresentarem as variações alélicas *-260T CD14*, *896A TLR4*, *1196C TLR4*, *2258G TLR2*, *2029C TLR2* e *-308G TNF-α* teriam uma resposta imune menos aberrante. Ao encontro disto, antes do presente trabalho haver sido realizado, o estudo de D'Avila *et al.*, com 87 indivíduos, mostrou o genótipo -260TT *CD14* como protetor para o desfecho de pacientes críticos, considerando a taxa de mortalidade.

3. OBJETIVOS

Com base na justificativa e hipótese apresentadas, os objetivos deste estudo foram:

1- Reproduzir o estudo de D'Avila *et al.* com um tamanho amostral mais expressivo, e verificar se o resultado proteção conferida ao genótipo -260TT *CD14* se confirma.

2- Investigar um conjunto de seis SNPs (-260C>T *CD14,* 2258G>A *TLR2,* 2029C>T *TLR2,* 896A>G *TLR4,* 1196C>T *TLR4 e* -308G>A *TNF-α*).

3- Verificar se há um efeito sinérgico da herança dessas variantes gênicas no desfecho dos pacientes em estado crítico de saúde, ligados a 4 genes na população pacientes críticos.

4- Correlacionar os resultados encontrados com a taxa de sobrevida.

CAPÍTULO 2

Immunological Investigations, 38: 1–15, 2009

ISSN: 0882-0139 print / 1532-4311 online
DOI: 10.1080/08820130903258818

informa
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The Influences of CD14 –260C>T Polymorphism on Survival in ICU Critically Ill Patients

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In order to analyze the effect of the two different versions of the cluster of differentiation 14 (CD14) receptor recognizing gene on survival, we determined the –260C>T single nucleotide polymorphism (SNP) frequencies in 514 critically ill patients. We compared the –260TT homozygotes with –260C allele carriers (–260CC and –260CT genotypes) and we demonstrated—260TT patients had the highest survival rate (82% *vs* 64%; $p < 0.001$; OR = 2.52, 95% CI = 1.43–4.46). We performed binary logistic regression, incorporating both –260C>T genotype groups and the main clinical predictors to exclude other risk factors that could influence the outcome form critical illness: higher age, APACHE II score, and length of stay at hospital, and the occurrence of sepsis and septic shock were risk factors to Intensive Care Unit (ICU) patient's mortality, but the –260TT genotype was protective factor toward survival ($p = 0.001$; OR = 3.08 95%CI = 1.54–5.98). Among septic and septic shock patients, –260TT genotype was also protective factor toward survival ($p = 0.001$; OR = 3.11 95%CI = 1.63–6.66 to septic patients, and $p = 0.001$; OR = 3.80 95%CI = 1.68–8.58 to patients with septic shock). Our results and our hypothesis suggest that the higher –260TT genotype frequency in ICU survivor patients is possibly explained by a beneficial effect on innate immunity signaling.

Keywords CD14, Polymorphism, Sepsis, Intensive Care Units, Longevity, Mortality.

INTRODUCTION

The CD14, a 53–55kDa glycoprotein, is a pattern recognition receptor with affinity to lipopolysaccharide (LPS) of Gram-negative bacteria (Ziegler et al., 1993) and also a receptor for peptidoglycans and lipoteichoic acid which are

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characteristic constituents of Gram-positive bacteria (Dziarsky et al., 1998; Fan et al., 1999). CD14 is expressed on the surface (mCD14) of myeloid (monocytes, macrophages and neutrophils) as well as non-myeloid (endothelial and epithelial) cells (Jersmann, 2005). CD14 is also abundant in a soluble form (sCD14), which apparently derives from the secretion of CD14 or from proteolysis of mCD14 (Le-Barillec et al., 1999; Schütt, 1999), and plays a crucial role in the immune function of cells that do not express mCD14 by enabling them to produce inflammatory cytokines in response to LPS challenge (Frey et al., 1992; Goyert et al., 1998; Landmann et al., 1996). Both mCD14 and sCD14 are pattern recognition receptors with an important role in innate immune response and facing Gram-negative (Landmann et al., 1995) and Gram-positive (Burgmann et al., 1996) sepsis.

A single nucleotide polymorphism (SNP) was detected in the CD14 promoter sequence, located in the locus 5q23-31; a transition from cytosine (C) to thymine (T) in the position –260 from the translation starting site of the gene (–260C>T; rs60313457) (Hubacek et al., 1999). This polymorphism is found near the recognition site for the transcription factor Sp1 and seems to have a significant role in CD14 regulation (Le Van et al., 2001; Zhang et al., 1994) since it can interfere quantitatively in the transcriptional capacity of the CD14 gene. In 2006, we evaluated the influence of the –260C>T CD14 SNP in a well-characterized sample of 85 critically ill patients from southern Brazil (D'Avila et al., 2006). Even with such small sample, the genotype and allele frequencies were similar to other studies with around one thousand subjects or more from different populations with the same European ethnic component (Kabesch et al., 2004; Koch et al., 2002; Nauck et al., 2002), and they were at Hardy-Weinberg equilibrium. With random genotype distribution for clinical characteristics at ICU patient admission, age, and length of hospital stay, we found that –260TT patients presented lower mortality when compared to the –260C carriers (Pearson chi-square unadjusted test $p = 0.002$; Logistic regression adjusted to confounding variables test $p = 0.016$; Kaplan-Meier Log-rank statistic $p = 0.042$). Other studies demonstrated that –260C allele was also associated with increased risk for severe sepsis (Barber et al., 2006) (228 subjects) or mortality (Barber et al., 2007) (233 subjects). Contrastingly, some studies revealed no association between –260C>T CD14 SNP and sepsis (Heesen et al., 2002; Hubacek et al., 2000) (204 patients and 247 control; 58 subjects) or infection diseases (Agnese et al., 2002; Rivera-Chavez et al., 2004) (77 patients and 39 control; 134 subjects).

Based on the above findings, it would be expected that polymorphic alterations on CD14 promoter sequence may influence the outcome of critically ill patients. Thus, in this work we tested whether the –260TT genotype would occur in ICU survivors patients more commonly than in decease patients in a larger well-characterized sample of 514 critically ill subjects.

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 1, 2002 and November 31, 2007. This general, non-pediatric Medical-Surgery Intensive Care Unit has 13 beds and receives 300 to 400 patients/year. We monitored patients daily during their entire ICU and post-ICU hospital stay. Patients were not eligible if they were diagnosed with HIV-infection, any known immunodeficiency, under immunosuppressive therapy, pregnant, or lactating. This sepsis-genotyping project was approved by our institutional Research Ethics Committee under protocols #03-01732 and #07-03990), and informed written consent or assent to participate was obtained from all patients or their surrogates.

Subjects and DNA Analysis

Genomic DNA was extracted from leucocytes by a standard method (Lahiri et al., 1991) and the –260C>T SNP genotyping was performed as previously described (D'Avila et al., 2006). To confirm that the 580bp PCR amplified product represented the targeted product, sequence analyses were obtained in MegaBase 1000 capillary DNA sequencer (Amersham Biosciences UK Ltd, Chalfont St Giles, Bucks, UK) using the same primers used for amplification. 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* CD14, exon5 DNA sequence (GI:4557416; GenBank accession number X74984 and U00699) 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. Laboratory technicians were blinded to phenotype whereas all clinical investigators were 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 (Bone et al., 1992). Sepsis was defined as 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 body core >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 met, but since all our subjects were at ICU with organ dysfunction, we called it as septic patients. 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 (Knaus et al., 1985) obtained on ICU admission day and used as an estimate for severity of disease. For daily organ dysfunction evaluation we used the *Sequential Organ Failure Assessment* (SOFA) (Vincent et al., 2002) score obtained on ICU admission day (SOFA-1) and in day 7 (SOFA-7), 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 were considered. Mortality was measured in days until death. Clinical endpoints of the study were discharge from the ICU (considered survivors), or death (considered non-survivors).

Statistical Analysis

Statistical calculations were performed using the SPSS 13.0 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 Mann–Whitney test. 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 backward stepwise logistic regression analysis (Wald method), incorporating patients with and without –260T 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 logistic model, we adopted as a criterion, an association between survivors and non-survivors and each independent variable at a significance level (p-value) lower than 0.25 (Moraes et al., 2005). We carried out a hazard function analysis (to mortality) by the Kaplan–Meier procedure. Based on the information that the –260TT homozygotes have an increased CD14 gene activation and produce more effective CD14 when compared with –260CC or –260CT subjects (Zhang et al., 1994), we use dominant-like model to –260C allele to perform our analysis. We believe that the

recessive or co-dominant models are not applicable to –260C allele since there is evidence for a cumulative transcriptional effect in one genotype. All reported p-values are two-tailed and considered statistically significant when 0.05 or less.

RESULTS

Subjects' Description

We obtained data from 514 patients that fulfilled a daily monitoring protocol from the first day of ICU admission until the endpoint of treatment. Table 1 illustrates a complete description of the ICU patients (n = 514) grouped according to the mortality at ICU: non survivors (31.7%; 163/514) and survivors (68.2%; 351/514). Demographic, clinical, and genetic characteristics were stated: the two groups had significant differences in the 13 parameters. In this unadjusted analysis, among ICU patients there were positive association with mortality the higher age, Apache II and SOFA scores, and length of stay at hospital, sepsis and septic shock occurrences, and genotype and alleles frequencies (all $p < 0.001$). The general genotypic frequencies in ICU sample were –260CC = 0.31 (157/514), –260CT = 0.49 (250/514), and –260TT = 0.20 (107/514), and the allelic frequencies were –260C = 0.54 (564/1028) and –260T = 0.46 (464/1028). These values did not differ from those expected by the Hardy–Weinberg model ($p = 0.920$). When we compared the three genotype groups (–260CC, –260CT, and –260TT) separately, we found significant differences between survivors and non-survivors ($p = 0.002$), and when we analyzed the survival between –260TT homozygote and non –260TT group (–260CC + –260CT genotypes) we noticed also a significant positive unadjusted association: the –260TT patients had the highest survival rate (82.2%; 88/107) when compared with non –260TT group [64.2%; 263/407 (–260CC: 37.6%; 59/157; –260CT: 34.0%; 85/250)] ($p < 0.001$; OR = 2.52, 95%CI = 1.43–4.46). The –260T *vs* –260C allele survival comparison also showed significant differences ($p = 0.001$; OR = 1.56, 95%CI = 1.18–2.06) (Table 1).

Clinical and Demographic Data According to Genotype Groups

We observed that the genotype groups were heterogeneous to Apache II and SOFA1 scores ($p < 0.05$), but not to gender, age, septic shock occurrence, or length of stay at ICU and at hospital ($p > 0.05$) (Table 2).

Logistic Regression Analysis

The non –260TT patients had a weak trend to more elevated sepsis rate ($p = 0.059$). Thus, we performed binary logistic regression to an adjusted analysis, incorporating both –260C>T genotype groups and the main clinical predictors

Table 1: Demographic, clinical, and genotypic data of critically ill patients by mortality.

Variables	All	Non Survivor	Survivor	p-value
Patients (n (%))	514	163 (31.7)	351 (68.3)	
Male (n (%))	275 (53.5)	87 (53.4)	188 (53.6)	0.968^{x2}
Age (years; mean (SD))	54.8 (20.1)	59.6 (18.3)	52.6 (20.5)	0.000ST
Admission cause - Medical (n (%))	431 (83.9)	139 (85.3)	292 (83.2)	0.550^{x2}
Admission cause - Surgical (n (%))	83 (16.1)	24 (14.7)	59 (16.8)	
APACHE II (mean (SD))	19.6 (7.8)	23.5 (7.0)	17.7 (7.5)	0.000ST
SOFA-1 (median (min-max))	6.0 (0–18)	8.0 (2–13)	6.0 (1–18)	0.000^{MW}
SOFA-7 (median (min-max))	5.0 (0–24)	6.0 (3–16)	5.0 (2–15)	0.000^{MW}
SOFA-15 (median (min-max))	5.0 (0–19)	6.0 (0–19)	4.0 (2–10)	0.000^{MW}
SOFA-29 (median (min-max))	4.0 (0–16)	5.5 (1–16)	3.0 (0–11)	0.000^{MW}
ICU LOS (median (min-max))	13 (0–259)	15 (0–259)	12 (1–125)	0.125ST
H LOS (median (min-max))	36 (1–277)	28 (3–277)	40 (1–242)	0.000ST
Sepsis (n (%))	342 (66.5)	147 (90.2)	195 (55.6)	0.000^{x2}
Septic shock (n (%))	243 (47.3)	126 (77.3)	117 (33.3)	0.000^{x2}
–260CC (n (%))	157 (30.5)	59 (36.2)	98 (27.9)	
–260CT (n (%))	250 (48.6)	85 (52.1)	165 (47.7)	0.002^{x2}
–260TT (n (%))	107 (20.8)	19 (11.7)	88 (25.1)	
–260CC+ –260CT (n (%))	407 (79.2)	144 (88.3)	263 (74.9)	0.000^{x2}
With –260C allele (n (%))	564 (54.8)	203 (62.3)	361 (51.4)	0.001^{x2}
With –260T allele (n (%))	464 (45.2)	123 (37.7)	341 (48.6)	

APACHE-II: Acute Physiology and Chronic Health Evaluation II; SOFA: Sequential Organ Failure Assessment; ICU: Intensive Care Unit; H: ICU plus hospital; LOS: Length of stay in days; 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 Non survivors and survivors patients; (a) –260CC+ –260CT genotypic group *versus* –260TT homozygotes; (b) –260TT+ –260CT genotypic group *versus* –260CC homozygotes.

Table 2: Patients' clinical and demographic data according to –260C>TCD14 genotype groups.

Variables	–260TT	Non –260TT	p-value
Patients (n (%))	107 (20.8)	407 (79.2)	NA
Male (n (%))	61 (57.0)	214 (52.6)	0.414^{x2}
Age (years; mean (SD))	60.2 (20.3)	62.8 (20.5)	0.244ST
Patients with sepsis (n (%))	63 (58.9)	279 (68.6)	0.059^{x2}
Patients with septic shock (n(%))	45 (42.1)	198 (48.6)	0.224^{x2}
APACHE II (mean (SD))	19.2 (8.4)	20.2 (7.3)	0.037^{MW}
SOFA-1 (median (min/max))[1]	6.0 (3/11)	7.0 (1/18)	0.043^{MW}
ICU LOS (days; median (min/max))	12.0 (0/65)	14.0 (1/259)	0.120^{MW}
H LOS (days; median (min/max))	33.0 (1/224)	36.0 (1/277)	0.949^{MW}

[1]Related to SOFA score at ICU admission. AN, anova test; APACHE-II, Acute Physiology and Chronic Health Evaluation II; ICU, intensive care unit; H, ICU plus hospital stay; LOS, Length of stay; MW, Mann–Whitney U-test; NA, not applicable; Non-260TT, –260C allele carrier; SD, standard deviation of the mean; SOFA, Sequential Organ Failure Assessment; ST, Student's *t*-test; v2, Pearson Chi-squared test.

to exclude other risk factors that could influence the outcome (Table 3) Taking all patients together (n = 514), step 2 (final) of the backward stepwise (Wald) method showed that higher age, APACHE II score, and length of stay at ICU or at hospital, and the occurrence of sepsis and septic shock were risk factor to

Table 3: Mortality outcome risk analysis by binary logistic regression of the backward stepwise (Wald) method: All critically ill patients (n = 514).

	Variable	Odds ratio (95% CI)	p
(A) Step			
Step 1	Age (+)	0.43 (0.19–0.95)	0.037
	Apache II (+)	0.22 (0.12–0.39)	0.000
	ICU LOS (+)	0.36 (0.20–0.64)	0.000
	Hospital LOS (+)	7.32 (4.11–13.04)	0.000
	SOFA 1 (+)	0.93 (0.53–1.64)	0.818
	Sepsis (+)	0.43 (0.19–0.95)	0.037
	Septic shock (+)	0.26 (0.13–0.375)	0.000
	–260TT (–)	3.10 (1.60–6.02)	0.001
Step 2	Age (+)	0.43 (0.19–0.95)	0.038
	Apache II (+)	0.22 (0.13–0.37)	0.000
	ICU LOS (+)	0.36 (0.20–0.95)	0.000
	Hospital LOS (+)	7.33 (4.11–13.05)	0.000
	Sepsis (+)	0.43 (0.20–0.950)	0.037
	Septic shock (+)	0.25 (0.14–0.46)	0.000
	–260TT (–)	3.08 (1.54–5.98)	0.001
(B) Step			
Step 1	Age (+)	0.36 (0.15–0.88)	0.025
	Apache II (+)	0.28 (0.15–0.51)	0.000
	ICU LOS (+)	0.44 (0.24–0.80)	0.008
	Hospital LOS (+)	6.11 (3.33–11.22)	0.000
	SOFA 1 (+)	1.02 (0.54–1.90)	0.950
	Septic shock (+)	0.25 (0.13–0.49)	0.000
	–260TT (–)	3.29 (1.62–6.66)	0.001
Step 2	Age (+)	0.36 (0.15–0.88)	0.025
	Apache II (+)	0.28 (0.16–0.50)	0.000
	ICU LOS (+)	0.44 (0.24–0.80)	0.008
	Hospital LOS (+)	6.12 (3.33–11.23)	0.000
	Septic shock (+)	0.25 (0.14–0.46)	0.000
	–260TT (–)	3.11 (1.63–6.66)	0.001
(C) Step			
Step 1	Age (+)	0.076 (0.20–0.29)	0.000
	Apache II (+)	0.33 (0.15–0.68)	0.003
	ICU LOS (+)	0.47 (0.25–0.95)	0.035
	Hospital LOS (+)	8.49 (4.07–17.68)	0.000
	SOFA 1 (+)	1.25 (0.58–2.68)	0.565
	–260TT (–)	3.77 (1.67–8.53)	0.001
Step 2	Age (+)	0.075 (0.019–0.28)	0.000
	Apache II (+)	0.35 (0.18–0.70)	0.003
	ICU LOS (+)	0.47 (0.23–0.94)	0.034
	Hospital LOS (+)	8.51 (4.08–17.73)	0.000
	–260TT (–)	3.80 (1.68–8.58)	0.001

(+)Related to mortality in ICU, (–) Related to survivor in ICU, CI, confidence of interval; SOFA, Sequential Organ Failure Assessment; –260TT, CD14 genotype and survive.

ICU patient's mortality, but the –260TT genotype was a protective factor towards survival (p = 0.001; OR = 3.08 95%CI = 1.54–5.98). Among septic and septic shock patients, step 2 (final) of the backward stepwise (Wald) method showed that the –260TT genotype was also a protective factor towards survival

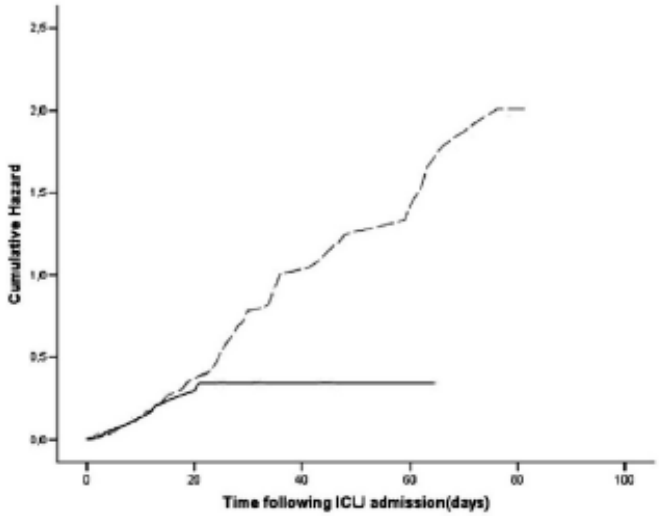

Figure 1: Cumulative hazard analysis for critically ill patients (n = 514) by the Kaplan–Meier procedure. TT (continuous line, n = 107) and non-TT (dotted line, n = 407) –260C>T CD14 genotype groups (Log-rank statistic, p = 0.037).

(p = 0.001; OR = 3.11 95%CI = 1.63–6.66 to septic patients, and p = 0.001; OR = 3.80 95%CI = 1.68–8.58 to patients with septic shock) (Table 3).

Kaplan-Meyer Analysis

We carried out a hazard function analysis by the Kaplan–Meier procedure using the –260TT genotype as a discriminating factor. Taking all patients together, we observed that patients carrying the –260TT homozygotes had a better outcome (Log-rank statistic, p = 0.037) when compared with those carrying the non –260TT genotype. Replicating what was observed in our first study with 85 patients (D'Avila 2006), both genotype groups (–260TT and non –260TT) reacted nearly equally until the third week after ICU admission (21 days). However, changes in the survival rate in favor of the –260TT homozygotes were observed after this period (Figure 1) The same analysis was conducted with patients with sepsis (n = 342) and septic shock (n = 243) and the survival distribution patterns were very similar although not statistically significant (sepsis p = 0.260 and septic shock p = 0.319; figures not shown).

Sepsis and Septic Shock Distribution

In our sample, the unadjusted genotype distribution analysis yielded just a trend when we compared the septic to the non-septic group (p = 0.053) (Table 4)

Table 4: Demographic, clinical, and genotypic data of critically ill patients by sepsis.

(A) Variables	All	With sepsis	Without sepsis	p
Patients (n (%))	514	342 (66.5)	172 (33.5)	
Male (n (%))	275 (53.5)	185 (53.9)	90 (56.6)	0.780^{x2}
Age (years; mean (SD))	54.8 (20.1)	61.9 (21.4)	64.1 (16.5)	0.153^{st}
Admission cause - Medical (n (%))	431 (83.9)	299 (87.2)	132 (77.2)	0.004^{x2}
Admission cause - Surgical (n (%))	83 (16.1)	44 (12.8)	39 (22.8)	0.004^{x2}
APACHE II (mean (SD))	19.8 (7.8)	21.2 (6.9)	16.1 (8.3)	0.282^{st}
SOFA-1 (median (min-max))	6 (0/18)	8 (1/18)	4 (1/12)	0.000^{MW}
SOFA-7 (median (min-max))	5 (0/24)	5 (2/16)	5 (3/19)	0.000^{MW}
SOFA-15 (median (min-max))	5 (0/19)	6 (0/19)	4 (3/9)	0.000^{MW}
SOFA-29 (median (min-max))	4 (0/16)	5 (0/8)	3 (0/8)	0.006^{MW}
ICU LOS (median (min-max))	13 (0/259)	44 (26/259)	34 (28/125)	0.003^{ST}
H LOS (median (min-max))	36 (1/277)	62 (9/277)	71 (35/242)	0.230^{ST}
Septic shock (n (%))	243 (47.3)	243 (70.8)	0 (0)	–
–260CC (n (%))	157 (30.5)	108 (31.5)	49 (28.7)	
–260CT (n (%))	250 (48.6)	172 (50.1)	78 (45.6)	0.153^{x2}
–260TT (n (%))	107 (20.8)	63 (18.4)	44 (25.7)	
–260CC + –260CT (n (%))	407 (79.2)	280 (81.6)	127 (74.3)	0.053^{x2}
With –260C allele (n (%))	564 (54.8)	388 (56.7)	176 (51.2)	0.718^{x2}
With –260T allele (n (%))	464 (45.2)	298 (43.3)	142 (48.8)	

–260T carriers: –260CC homozygotes and –260CT heterozygotes to –260C>T CD14 SNP; –260TT patients: –260TT homozygotes; APACHE-II: Acute Physiology and Chronic Health Evaluation II; SOFA: Sequential Organ Failure Assessment; ICU: Intensive Care Unit; 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 non-survivors and survivors patients; (a) –260CC + –260CT genotypic group *versus* –260TT homozygotes; (b)–260TT+ –260CT genotypic group *versus* –260CC homozygotes.

DISCUSSION

In our study with 514 ICU critically ill patients we compared the –260TT CD14 homozygotes with non–260TT genotype group (–260CC and –260CT) and demonstrated that the –260TT genotype was a protective factor towards survival (Pearson chi-square unadjusted test $p < 0.001$; Logistic regression adjusted to confounding variables test $p = 0.001$; Kaplan-Meier Log-rank statistic $p = 0.037$).

Some studies (but not all) (Heesen et al., 2001) have shown the –260TT homozygotes with increased CD14 expression (levels of sCD14, monocyte mCD14 density, and/or CD14 mRNA) in comparison to –260C allele carriers (Aguiar et al., 2008; Baldini et al., 1999; Karhukorpi et al., 2002; Koenig et al., 2002; Lin et al., 2007; Zhang et al., 1994). These data led us to believe that CD14 expression is influenced, at least in part, by the genetic inheritance of –260C>T CD14 SNP.

Monocyte mCD14 density and sCD14 serum levels are significantly higher in early septic patients than in normal controls, and in the peripheral blood mononuclear cells *in vitro* experiment. Moreover mCD14 density is significantly increased after LPS challenge (Aguiar et al., 2008). Accordingly, other

experiments demonstrate that an increased concentration of sCD14 was found in patients with infection and/or sepsis (Brunialti et al., 2006; Burgmann et al., 1996; Carrillo et al., 2001; Glück et al., 2001; Hiki et al., 1998; Landmann et al., 1995, 1996). These findings are suggestive that exposure to inflammatory milieu and/or infection up regulates CD14 expression, although mCD14 density was observed to be significantly lower in patients than in controls (Brunialti et al., 2006; Ertel et al., 1995; Glück et al., 2001; Hiki et al., 2006) or no difference (Calvano et al., 2003; Carrillo et al., 2001; Tsujimoto et al., 2005). It should not be ruled out that a significant change in mCD14 expression could be occurring in the early phase of sepsis, and it would affect the subsequent sCD14 levels. To explain that, Glück et al. valued mCD14 and sCD14 expression levels at the entry of their study and in the last sample from patients prior to death or hospital discharge, concluding that higher sCD14 levels may be beneficial in sepsis and that persistently reduced mCD14 expression could be a marker for severity of disease in critically ill patients (Karhukorpi et al., 2002). Hence, it is likely to expect that, in one hand, CD14 expression would be increased during the critical circumstances and, on the other hand, that persistently enhanced CD14 expression contributes to a favorable outcome.

Regarding the influence of –260C>T CD14 SNP upon monocyte mCD14 density or sCD14 serum levels, it was reported that this promoter SNP interferes quantitatively in the gene transcriptional activity (Le Van et al., 2001; Zhang et al., 1994), but the correlation between CD14 polymorphic inheritance and phenotype/symptoms is not clear yet. While some studies revealed no association between –260C>T CD14 SNP and sepsis (Barber et al., 2007; Hubacek et al., 2000) or infection diseases (Agnese et al., 2002; Heesen et al., 2002), others observed that the –260C>T genotype confers susceptibility to sepsis (Lin, 2004), severe sepsis (Kabesch, 2004), septic shock, and mortality (Barber et al., 2006; D'Avila et al., 2006; Gibot et al., 2002).

It was not unnoticed to us that much of this controversy may derive from methodological disparities among studies concerning the time elapsed between the onset of sepsis and the analysis of patient material, since the time between sepsis diagnosis and sample obtaining can be an important factor influencing these different CD14 expression results. Our results avoid this interference since rely solely on genotypes, showing significant differences in genotypic frequencies between survivors and non-survivors ICU patients. In the same way, the unadjusted genotype distribution analysis had only a trend when we compared the septic to the non-septic group ($p = 0.053$). Thus, data suggest that the CD14 polymorphism had insufficient power to affect our ICU patient susceptibility to infection, but it seems to be applicable as a marker for survival/mortality in critically ill patients.

Our suggestion is that –260TT patients had more accessibility to provide CD14 during the evolution of the illness. We also suggest, in conformity

with Glück et al. and Aguiar et al., that an increase in the CD14 concentration in –260TT patients could be a protective factor that helps the immune system to improve the balance of pro-inflammatory and anti-inflammatory actions (Brunialti et al., 2006; Heesen et al., 2001). Similar conclusions were observed in another study where the –260CC was related to increase for death risk in patients with burn injury (Barber et al., 2006).

We did not evaluate the effect of the CD14 genotype by segregating patients accordingly to bacteria type or disease group due to the fact that CD14 recognizes Gram-negative as well as Gram-positive bacteria (Gupta et al., 1996; Wright et al., 1990). However, we acknowledge one major limitation in our study, as follows: neither a haplotyped-based nor a cluster-based CD14 gene approach were performed. Single-marker studies are limited by the fact that a particular gene polymorphism is likely to be influenced by *loci* in linkage, and just be a marker for some unidentified variables. Despite of this, our study was able to detect a significant effect of –260C>T under ICU outcome, showing that this unique SNP studied may be of biological relevance. In addition, we believe our methods are reliable since we used a quality control system to ensure genotyping precision (sequencing verification of the DNA amplified fragment, black controls, and repetitions), state-of-art study design (double-blinded approach), and universal standards for definitions of sepsis and septic shock (worldwide accepted scores to determine organ dysfunction and illness severity). All the above considered renders a plausible effect of the gene product in the outcome of our critically ill patients.

Last, we propose that the further SNP-array investigations should include the –260C>T CD14 SNP alone or in combination with other functionally relevant mutations. Broader advanced studies including additional candidate CD14 SNPs and genes such as the Toll-like Receptor (TLR) 4, TLR2, or TNF-alpha genes could also help to refine the understanding about ICU risk. We are currently investigating some of these possibilities.

CONCLUSIONS

In conclusion, we showed that the –260C>T CD14 SNP is a protective factor towards survival in critically ill patients; there was higher frequency of survivors in –260TT homozygotes. Our results and our hypothesis suggest that the higher –260TT genotype frequency in ICU survivor patients is possibly explained by an effect on innate immunity signaling.

ACKNOWLEDGMENTS

We thank L.B. Miranda, C.B.C. Bonorino, and M.P. Garrido for their suggestions and L.R. Fraga, for technical assistance. This study was financed by Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq

(process # 505536/2002-8), the Programa de Bolsa Pesquisa para Alunos da Graduação – Edital BPA PUCRS 2007–2008, and Faculdade de Biociências, PUCRS. The study is part of the Doctor's Degree thesis of the first author who had a fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES, Brazil.

Financial Support Used for the Study, Including any Institutional Departmental Funds:

This study was financed by Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (process# 505536/2002-8), the Programa de Bolsa Pesquisa para Alunos da Graduação – Edital BPA PUCRS 2007-2008, Faculdade de Biociências – PUCRS, and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES, Brazil.

Declaration of Interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Title of the manuscript: The synergy of -260TT *CD14* and -308GG *TNF-a* genotypes in critically ill patients' survival.

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

This study was financed by Fundação de Amparo à Pesquisa do Rio Grande do Sul and Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (FAPERGS/MS/CNPq/SESRSEFP00000829).

ABSTRACT

Background:

The susceptibility to adverse outcome from critical illness and mortality varies due to different degrees of immunological response. In our previous work we showed that the -260C>T single nucleotide polymorphism (SNP) in cluster of differentiation 14 (*CD14*) was a protective factor towards survival in critically ill patients. The -260TT homozygotes have increased *CD14* expression and it may be beneficial during critical circumstances, hence a persistently enhanced *CD14* expression contributes to a favorable outcome from ICU. Recent literature suggests that the analysis of a lot of polymorphic genetic markers is more informative than the analysis of a single polymorphism. SNPs in other genes that encode *CD14* synergic proteins could also be informative in patients with critical health condition. We verified whether the shared inheritance of Toll-like receptors 2 and 4 (*TLR2* and *TLR4*) and tumor necrosis factor α (*TNF-α*) allelic variants may act in synergy with -260C>T *CD14* SNP on the outcome from critical conditions.

Methods:

We monitored 524 critically ill patients from South Brazilian patients daily from the ICU admission day to their discharge from hospital, or death, measuring clinical conditions. We analysed the SNPs 2029C>T *TLR2*, 2258G>A *TLR2*, 896A>G *TLR4*, 1196C>T *TLR4*, and -308G>A *TNF-α*.

Results:

Our results revealed that *TLR2*, *TLR4* or *TNF-α* SNPs alone did not show a significantly remarkable role in the outcome from critical illness. However, when we performed a combined analysis with the *CD14* inheritance, we detected a significant higher survivor rate in *-260TT CD14/*-308GG *TNF-α* double-homozygote group. In the adjusted analysis with double-genotype variable and the main clinical predictors to mortality, we observed that -260TT *CD14/*-308GG *TNF-α* double-genotype was a significant protective factor towards survival. Connected to the beneficial effect of -260TT *CD14*, the -308GG *TNF-α* genotype was protector against the reported overexpression of *TNF-α* caused by *-308A* rare allele.

Conclusion:

Our results support the hypothesis that the interaction between -260TT *CD14* and -308GG *TNF-α* functional SNPs may be influencing the outcome of critically ill patients.

KEY WORDS

CD14; *TNF-α*; Critical Care; SNP, Mortality

INTRODUCTION

The mortality rate among ICU patients varies despite efforts [1]. This may be due, at least in part, to the genetic variables of each patient facing critical illness. Higher expression of cluster of differentiation 14 (*CD14*) gene levels may be beneficial in ICU patients and a persistently reduced *CD14* gene expression is also a marker for the severity of disease in critically ill patients [2]. The -260C>T SNP in *CD14* gene interferes quantitatively in its transcriptional activity and has an influence on monocyte m*CD14* density and/or s*CD14* serum levels [3, 4]. In our two previous works we showed that the -260C>T *CD14* SNP was a protective factor towards survival in critically ill patients; there was a higher frequency of survivors in -260TT homozygotes [5, 6]. Simultaneously, it was observed that -260CC increased the death risk in critically ill patients with burn injury [7, 8]. In conformity with Glück *et al*. and de Aguiar *et al*. our suggestion was that the *CD14* level increase in -260TT patients could help the immune system improve the balance of pro-inflammatory and anti-inflammatory actions [2, 9]. Literature shows that polymorphic variants in genes that encode proteins which have synergistic action with *CD14* could also be informative on critical conditions [10]. Among the synergic proteins we notice Toll-like receptors 2 and 4 (*TLR2* and *TLR4*) and tumor necrosis factor α (*TNF-α*). *TLR2* and *TLR4* play an important role in the recognition of pathogen-derived pattern ligands such as lipopolysaccharide (LPS), peptidoglycan, lipoarabinomannan and lipoproteins [10, 11, 12]. When LPS bounds to lipopolysaccharide binding protein (LBP) and to *CD14* it initiates signal transduction cascades inside cells through *TLR4* or *TLR2*, leading to the translocation of NF-κB into the nucleus where it regulates the expression of pro-inflammatory cytokines [12-14]. Activated macrophages secrete pro-inflammatory cytokines like *TNF-α* which initiates the cascade of immunity response [15]. Different genetic epidemiological studies focused on genes involved inflammatory pathway have demonstrated a significant genetic influence in the outcome of critically ill patients [16, 17]. However, the majority of these studies examined the isolated effect of each gene; only few studies have combined the analysis of multiple SNPs in the same population [18, 19]. In the present work, we conducted a study with 524 critically ill patients to determine whether the shared inheritance of *TLR4*, *TLR2*, and *TNF-α* allelic variants may act in synergy with -260C>T *CD14* SNP on the outcome of critical patients.

METHODS

Design, subjects, and approval

This single centre observational retrospective cohort study was conducted with data from random patients admitted to the Intensive Care Unit (ICU) of the São Lucas Hospital (HSL), Brazil, between March 1st, 2002 and December 31st, 2007. The ICU-HSL is a general non-pediatric Medical-Surgery Intensive Care Unit with 13 beds, which receive about 300-400

patients per year. We worked on the archived DNA collection from critically ill patients which was monitored daily during their entire ICU stay. Patients were not eligible if they were diagnosed with HIV-infection, with known immunodeficiency, taking immunosuppressive drugs, pregnant, or lactating. All subjects were from Southern Brazil which is composed of a singular genetic background: majority of subjects with European origin (Portuguese, Italians, Spanish, and Germans ancestry) and a small amount of individuals with African traits contributing to their genetic pool [20, 21]. This ICU-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 #08-04199), and the informed written consent or assent to participate in was obtained from all subjects or patients' surrogates.

Phenotyping

The patients were diagnosed with 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 [22]. For illness severity evaluation we used the APACHE-II (Acute Physiology and Chronic Health Evaluation II) score [23] 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) [24] 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 ICU. Temporal variation comprised length of stay (LOS) in ICU. Mortality was measured in days until death in total ICU stay: clinical endpoints of the study were discharge from the ICU (considered survivors), or death (considered non-survivors). For those patients with multiple ICU admission during the study period, only data from the first entrance was considered. All clinical data was collected and verified by ICU physicians with control ensure.

Genotyping

Genomic DNA was extracted from leucocytes by a standard method [25], primers, probes and restriction enzymes are shown in Table 1.

The -260C>T *CD14* (rs2569190; NT_029289.11:g.1175843A>G), 2029C>T *TLR2* (no rs) and 2258G>A (rs5743708; p.Arg753Gln; NT_016354.19:g.79174038G>A) *TLR2* genotyping was performed as previously described [7, 26]. *TNF-α* -308G>A SNP (rs1800629; NT_007592.15:g.31483031G>A) and *TLR4* 896A>G & 1196C>T SNPs (rs4986790; p.Asp299Gly; g.13843A>G) and 1196C>T (rs4986791; p.Thr399Ile; g.14143C>T), based on Arbour *et al*. [27], was analyzed by Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) analysis in a PTC-100 thermocycler (MJ Research, Watertown, MA, USA). The *TNF-α* 116 bp PCR product was obtained from a 25µl reaction mix containing 10-100 ng DNA, 1 µM each primer, 0.4 mM dNTP, 1.5 mM $MgCl_2$ and 1 U Taq polymerase in Taq 1x Buffer (Invitrogen-Life Technologies, California, USA). The reaction was carried out in a PTC-100 thermocycler (MJ Research, Watertown, MA, USA) as follows: 95°C for 2 minutes; 35

cycles of 95°C for 30 s, 60°C for 15 s and 74°C for 15 s; and 74°C for 10 minutes for final extension. The PCR amplified product (12µl) was cleaved in an appropriate buffer with 10U of the *Nco*I (5'-C/CATGG-3'; GibcoBRL®-Life Technologies™, Rockville, MD, USA) in a total volume of 15µl at 37ºC for 16 hours, and digested or undigested samples were visualised by electrophoresis in 3% agarose gel with GelRed Nucleic Acid Stain (Biotium Inc. Hayward, CA).

For the *TLR4* both DNA sequences were amplified in a 25 µL reaction containing 10-100 ng of DNA, 0.2 mmol/L of each dNTP, 2 mmol/L of $MgCl_2$ and 1.25 U Taq DNA Polymerase in Taq Buffer (Invitrogen-Life Technologies, California, USA). Cycling conditions for both polymorphisms were 95°C for 5 min, followed by 36 cycles at 95°C for 30s, 55°C for 30s and 72°C for 30s; and, finally, a 5 min extension at 72°C. The amplified PCR products (20 µL) were cleaved, in an appropriate buffer, with 5U of the NcoI (New England Biolabs™, USA) for the 896A>G SNP; and with 5U of MspI (New England Biolabs™, USA) for 1196C>T SNP in a total volume of 25µL, at 37ºC for 4 hours. The restriction digest fragments were visualized on a 2% agarose gel.

At least 10% of the samples were subjected to a second and independent PCR-RFLP analysis cycle to confirm the genotypes. In order to confirm that the PCR amplified products really represented the targeted products, 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. These sequences were submitted to online BLASTn alignments (at http://www.ncbi.nlm.nih.gov/BLAST/), and we found consensus with the *Homo sapien* toll-like receptor transcript variant 4 sequence (GI: 88758616; NM138554.3). The same was done to *TNF-α* PCR products with match with *Homo sapiens* tumor necrosis factor-α gene, promoter region (GenBank accession X02910). Blank control wells were always used to test contamination of the PCR reagents. All the personnel involved in patient care were blind to the selection process and genotyping results.

Statistics

Statistical calculations were performed using the SPSS 13.0 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 analysed as non-parametric using the Mann–Whitney test. For categorical data, we used the Pearson Chi-squared test. For the inclusion of variables in the multivariate logistic model, we adopted as a criterion, an association between survivors, non-survivors and each independent variable at a significance level (P-value) lower than 0.25 [28]. We carried out a hazard function analysis (to mortality) by the Kaplan–Meier procedure. To test Hardy–Weinberg equilibrium (HWE), the Chi-squared test was used. HWE analysis was performed for each SNP by comparing the detected genotype distribution with the theoretical distribution estimated on the basis of the SNP allelic frequencies.

RESULTS

During March 1st, 2002 to December 31st, 2007 was included in this study a total of 524 critically ill adult patients from Southern Brazil. Of these 524 patients included in the study, 105 patients were subtracted from a former study duo to difficulty for amplification for all six SNPs, and included another 105 patients that we could amplify all the fragments for the six SNPS [6]. The general frequencies of rare alleles were: *CD14 -260T*= 0.47 (483/1044); *TLR4 896G*= 0.05 (54/1048) and *1196T*= 0.13 (143/1048); *TLR2 2029T*= 0 and *2258A*= 0; *TNF-α -308A*= 0.14 (152/1044). All genotypes were in Hardy-Weinberg equilibrium. No associations were found between genotypes, sex, age, APACHE II or SOFA scores, ICU length of stay and sepsis or septic shock rates. In total 174 (33%) died during ICU hospitalization. The patient's phenotypic and genotypic data according in-ICU survivor rate are in Table 2.

As expected from our previous work [5, 6], by univariate analysis, the survivor rate in this 524 patient's group was confirmed to be inversely associated with age, SOFA scores, and length of stay at ICU, sepsis, and septic shock and it was directly associated with *CD14* -260TT homozygotes (P=0.045; OR=1.60; 95% CI=0.98-2.61). We did not detect any isolated association between the other tested SNPs and outcome. When we analyzed -260TT genotype combined with other SNPs, we observed a higher survivor rate detected in double-homozygote patients (P=0.039; OR=1.79; 95% CI=0.99-3.28) (Table 3). By Pearson correlation analysis, we observed that the presence of genotype -260TT and the double homozygous -260TT *CD14*/-308GG *TNF-α* was directly related to a higher survival rate among critically ill patients (P = 0.042, P = 0.037 respectively). To make sure that this relationship is confirmed, we also performed binary logistic regression to an adjusted analysis, incorporating -260TT *CD14*/-308GG *TNF-α* double-genotype variable and the main clinical predictors to exclude other risk factors that could influence the outcome (Table 4). Taking all patients together (n=524), step 3 (final) of the backward stepwise (Wald) method showed that higher age, APACHE II score, and the occurrence of sepsis/septic shock were risk factor to ICU patient's mortality, but the -260TT *CD14*/-308GG *TNF-α* double-genotype was a protective factor towards survival (P=0.046; OR=1.89; 95% CI=1.01-3.53). When we carried out a hazard function analysis by the Kaplan–Meier procedure using the -260TT *CD14*/-308GG *TNF-α* double-genotype variable as a discriminating factor, we observed that patients carrying the -260TT *CD14*/-308GG *TNF-α* double-genotype had, in fact, a better outcome (Log-rank statistic; P=0.024) (Figure 1).

DISCUSSION

Genetics is important to understand complexity of the human immune system and how our organism responds to extreme situation such as critically care condition [29]. In this study, we detected a significant higher survivor rate in -260TT *CD14*/-308GG *TNF-α* double-homozygote critically ill patients. Our combined analysis with more than one SNP was especially interesting because it demonstrated that the synergic effect of these two gene variations was stronger than -260TT *CD14* or -308GG *TNF-α* effects when they were studied alone. In addition, when we performed the adjusted analysis, incorporating the double-genotype variable and the main clinical predictors to mortality, we observed that -260TT *CD14*/-308GG *TNF-α* double-genotype was a protective factor towards survival.

In the non-combined analysis with 524 ICU critically ill patients we compared the -260TT *CD14* homozygotes with non -260TT genotype group (-260CC and -260CT) and confirmed that the -260TT genotype was a protective factor towards survival. Some studies (but not all) [30] have shown the -260TT homozygotes with increased *CD14* expression (levels of s*CD14*, monocyte m*CD14* density and/or *CD14* mRNA) in comparison to *-260C* allele carriers [9, 31-33]. It was demonstrated that higher s*CD14* levels may be beneficial and persistently reduced m*CD14* expression could be a marker for the severity of disease in critically ill patients [2]. These data led us to believe that *CD14* expression is influenced, at least in part, by the genetic inheritance of -260C>T *CD14* SNP. Hence, it is likely to expect that *CD14* expression would be increased during the critical circumstances and that persistently enhanced *CD14* expression contributes to a favorable outcome [2]. Regarding that the influence of -260C>T *CD14* promoter SNP on transcriptional rate of this gene [3, 4], our suggestion was that -260TT patients had more accessibility to provide *CD14* during the evolution of the illness. An increase in the *CD14* concentration in -260TT patients could be a protective factor that helps the immune system to improve the necessary balance of pro-inflammatory and anti-inflammatory actions [30, 34].

Our non-combined analysis revealed no association between -308G>A *TNF-α* genotypes and an outcome from critical illness. The -308G>A *TNF-α* SNP alone did not play a major role in the outcome from critical illness. Considering that the susceptibility to adverse outcome from critical illness varies dramatically due to different degrees of immunological response, an inappropriate expression or overexpression of *TNF-α* could lead to a decline of the patient condition [35-37]. The *-308A* rare allele of *TNF-α* gene cause a differential binding of nuclear factors and leads to a six to sevenfold increase in its inducible level of transcription [38,39]. Thus, the presence of *-308A* allele could be dangerous during the critical circumstances. However, when studied alone, the presence of the *-308A TNF-α* allele (genotype group -308GA and -308AA) was not sufficiently strong enough to lead critically ill patients into susceptibility to adverse outcome. For our large sample, at least, two factors may have contributed to this result. First, the *TNF-α* gene is in the highly polymorphic 6p21 locus, within the class III region for major histocompatibility complex (MHC), between lymphotxin-α (TNF-β) and lymphotxin-β

genes [40]. Strong linkage disequilibrium among *TNF-a*, and these other alleles create established haplotypes which affect differently the phenotype. In this case, the single -308G>A SNP analysis may have very low power to detect the real and particular effect of each -308G>A *TNF-a* allele. Second, in addition to *TNF-a*, there are other molecules involved in the immune response (like *CD14*) encoded by genes located on other chromosomes, which can interfere in the inflammatory field causing, once again, misleading conclusions in a single SNP analysis. The combined contribution of multiple genes and environment factors affect the onset, evolution, and outcome of complex diseases. Each gene, or gene variation, contributes with small and cumulative effects on the final phenotype. Because of this, it would be acceptable that each one of these small effects would not be seen through single association studies [41].

In association studies, combined analyses are more strongly informative about the synergistic effect of inheritance. For example, a great synergistic relationship among genes on the phenotype was notice by Nysquist and cols. that demonstrated a simultaneous effect of MCP-1 and CCR2 gene variation inheritance on the carotid atherosclerosis risk [42]. More similar studies can be seen in Seitsonen and cols. and Gigante and cols. [18, 19]. The combined detection of independent segregated SNPs may provide a deeper insight about whether the genes simultaneously interact and whether this interaction can affect the phenotype. Here, we worked with over 500 patients, all of them being in critically conditions. We performed our comparisons always among ICU patients; we did not used healthy subjects as control group to exclude the crucial influence of environmental exposure. This study design may explain why our population was able to demonstrate the pooled effect of -260TT *CD14*/-308GG *TNF-a* inheritance on ICU patient outcome with statistical significance.

Accepting the reported functional effects of these SNPs on *CD14* and *TNF-a*, the -260TT *CD14*/-308GG *TNF-a* patients would have persistently enhanced *CD14* expression and standardized *TNF-a* transcription levels.

In conclusion, our results support the hypothesis that the interaction between -260TT *CD14* and -308GG *TNF-a* functional SNPs may be influencing the patients outcome from critical health conditions.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

PRVF, TJB, DDP, CSF, PG, and HS developed and performed the genetic analysis. PRVF and DDB, FJP, CSA, were responsible for conception, design, data collection and statistical analysis. All authors participated in the writing of the manuscript. All authors have read and approved the final version of the manuscript.

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Table 1: *-260 C>T CD14,* -308G>A *TNF, 896 A>G and* 1196C>T *TLR4, 2029 C>T and 2258 G>A TLR2 SNPS.*

Gene	SNP	Sense primer	Antisense primer	Probe/Restriction enzyme	Conditions
CD14	-260 C>T	TTGGTGCCAA CAGATGAGGT TCAC	TTCTTTCCTACACAGC GGCACCC	*Hae*III	95°C for 2 minutes, followed by 35 cycles at 92.3°C for 40 seconds, at 59.5°C for 35 seconds, and at 71.5°C for 50 seconds. The final extension step was prolonged to 5 minutes
TNF-α	-308G>A	AGGCAATAGG TT TTGAGGG-CCAT	ACACTCCCCATCCTCC CTGCT	NcoI (5'C/CATGG3`)	95°C for 2 minutes; 35 cycles of 95°C for 30 s, 60°C for 15 s and 74°C for 15 s; and 74°C for 10 minutes for final extension
TLR2	2029C>T	GCCTACTGGG TGGAGAACCT	GGCCACTCCAGGTAG GTCTT	Not used	95°C for 10 minutes; 35 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 30 s; and 72°C for 5 minutes for final extension
	2258G>A			Not used	
TLR4	896A>G	GATTAGCATA CTTAGACTACT ACCTCCATG	GATCAACTTCTGAAAA AGCATTCCCAC	NcoI (5'C/CATGG3`)	95°C for 5 min, followed by 36 cycles at 95°C for 30s, 55°C for 30s and 72°C for 30s; and, finally, a 5 min extension at 72°C.
	1196C>T	GGTGAGTGTG ACTATTGAAA GGGTAAAAG	GAAGCTCAGATCTAAA TACTTTAGGCCG	MspI (5'C/CGG3')	

*100 to 200 pmol of genomic DNA; sense primer 5' -3'; Antisense primer 3'-5'

Table 2: Phonotypical and genotypic data of critically ill patients according in-ICU survivor rate.

Variables	All	Non Survivor	Survivor	P value
Patients [n (%)]	524 (100)	174 (33.2)	350 (66.8)	-
Male [n (%)]	280 (53.4)	92 (52.9)	188 (53.7)	0.856^{X2}
Age [Years; mean (SD)]	54.7 (20.0)	62.9 (21.3)	60.7 (19.8)	0.011^{ST}
APACHE II [mean (SD)]	19.6 (7.8)	22.2 (7.4)	18.8 (7.1)	0.183^{ST}
SOFA-1 [median (min-max)]	6.0 (0-18)	8.0 (3-13)	6.0 (1-18)	0.000^{MW}
SOFA-7 [median (min-max)]	5.0 (0-24)	6.0 (3-16)	5.0 (2-15)	0.000^{MW}
SOFA-15 [median (min-max)]	5.0 (0-19)	7.5 (0-19)	5.0 (2-11)	0.000^{MW}
SOFA-29 [min-max)]	4.0 (0-16)	7.5 (2-16)	3.0 (0-11)	0.000^{MW}
ICU LOS (median (min-max)]	13.5 (0-259)	42.5 (26-259)	38.0 (28-125)	0.007^{ST}
Sepsis [n (%)]	351 (67.0)	157 (90.2)	194 (55.4)	0.000^{X2}
Septic shock [n (%)]	252 (48.0)	135 (77.5)	117 (33.4)	0.000^{X2}
CD14				
-260TT [n (%)]	117 (22.3)	30 (17.2)	87 (24.8)	0.045^{X2}
-260CC+-260CT [n (%)]	405 (77.2)	144 (82.7)	261 (74.5)	
TLR2				
2029CC [n (%)]	524(100)	174(100)	350(100)	**-**
2029TT+2029CT [n (%)]	-	-	-	
2258GG [n (%)]	524(100)	174(100)	350(100)	**-**
2258AA+2258GA [n (%)]	-	-	-	
TLR4				
896AA [n (%)]	470(89.6)	156 (89.6)	314 (89.1)	0.983^{X2}
896AG+896GG [n (%)]	54(10.3)	18 (10.3)	36 (10.2)	
1196TT [n (%)]	20(3.81)	10 (5.7)	10 (2.8)	0.104^{X2}
1196CC+1196CT [n (%)]	504(96.1)	164 (94.2)	340 (97.1)	
TNF-a				
-308GG [n (%)]	376(71.7)	122 (70.1)	254 (72.5)	0.695^{X2}
-308AA+-308GA [n (%)]	146(27.8)	50 (28.7)	96 (27.4)	

n: number; SD: Standard Deviation of the mean; APACHE-II: Acute Physiology and Chronic Health Evaluation II; SOFA: Sequential Organ Failure Assessment; ICU: Intensive Care Unit; LOS: Length of stay in days; X2: Pearson Chi-Square test; ST: Student's t-test; MW: Mann-Whitney U-test; P-value describes a comparison between non-survivors and survivor patients.

Table 3: Gene-gene interaction of critically ill patients according in-ICU survivor rate.

	All (524)	Non Survivor (174)	Survivor (350)	P value
-260TT/896AA [n (%)]	105	27 (15.6)	78 (22.3)	0.073^{x2}
Remaining Genotype Groups [n (%)]	419	146 (84.4)	272 (77.7)	
-260TT/1196CC [n (%)]	92	28 (16.1)	64 (18.4)	0.516^{x2}
Remaining Genotype Groups [n (%)]	430	146 (83.9)	284 (81.6)	
-260TT/896AA/1196CC [n (%)]	86	25 (14.4)	61 (17.5)	0.366^{x2}
Remaining Genotype Groups [n (%)]	437	149 (85.6)	288 (82.5)	
-260TT/-308GG [n (%)]	78	18 (10.4)	60 (17.2)	0.039^{x2}*
Remaining Genotype Groups [n (%)]	442	155 (89.6)	288 (82.7)	
-260TT/896AA/-308GG [n (%)]	68	16 (09.4)	52 (15.0)	0.073^{x2}
Remaining Genotype Groups [n (%)]	452	156 (90.6)	296 (85.0)	
-260TT/1196CC/-308GG [n (%)]	60	17 (09.8)	43 (12.4)	0.406^{x2}
Remaining Genotype Groups [n (%)]	460	155 (90.2)	305 (87.6)	
-260TT/896AA/1196CC/-308GG [n (%)]	55	15 (08.7)	40 (11.4)	0.338^{x2}
Remaining Genotype Groups [n (%)]	466	157 (91.3)	309 (88.5)	

n: number; P-value describes a comparison between non-survivors and survivor patients by gene-gene interaction; Pearson Chi-Square test; *: P<0.05.

Table 4: Mortality outcome risk analysis by binary logistic regression of the backward stepwise (Wald) method: All critically ill patients (n=524).

(A) Step	**Variable**	**Odds Ratio (95% CI)**	**P value**
Step 1	Age (+)	0.61 (0.40-0.93)	0.024*
	APACHE II (+)	0.42 (0.27-0.66)	0.000***
	ICU LOS (+)	0.83 (0.50-1.80)	0.886
	SOFA 1 (+)	0.95 (0.51-1.33)	0.446
	Sepsis/Septic shock (+)	0.18 (0.11-0.21)	0.000***
	-260TT*CD14*/-308GG*TNF-α* (-)	1.88 (1.00-3.52)	0.048*
Step 2	Age (+)	0.61 (0.40-0.93)	0.022*
	APACHE II (+)	0.42 (0.27-0.66)	0.000***
	ICU LOS (+)	-	-
	SOFA 1 (+)	0.83 (0.51-1.33)	0.448
	Sepsis/Septic shock (+)	0.18 (0.11-0.29)	0.000***
	-260TT*CD14*/-308GG*TNF-α* (-)	1.88 (1.00-3.51)	0.048*
Step 3	Age (+)	0.61 (0.40-0.93)	0.022*
	APACHE II (+)	0.40 (0.26-0.61)	0.000***
	ICU LOS (+)	-	-
	SOFA 1 (+)	-	-
	Sepsis/Septic shock (+)	0.16 (0.10-0.26)	0.000***
	-260TT*CD14*/-308GG*TNF-α* (-)	1.89 (1.01-3.53)	0.046*

(+) Related to mortality in ICU; (-) Related to survivor in ICU; CI, confidence of interval; APACHE-II: Acute Physiology and Chronic Health Evaluation II; SOFA: Sequential Organ Failure Assessment; ICU LOS: Length of stay in days at Intensive Care Unit; *: P<0.05; ***: P<0.001.

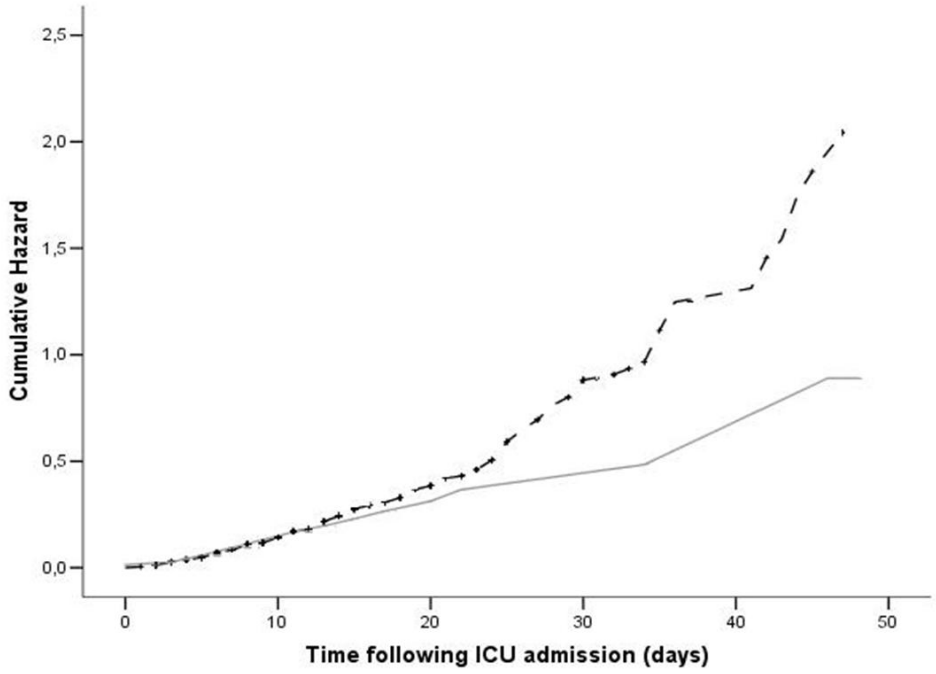

Figure 1: Cumulative hazard analysis for critically ill patients by the Kaplan–Meier procedure. -260TT *CD14*/-308GG *TNF-α* (continuous line) and others SNPs (dotted line) *−260C>T CD14* e -308G>A *TNF-α* genotype groups (Log-rank statistic; P=0.024).

CAPÍTULO 3

CONSIDERAÇÕES FINAIS E CONCLUSÕES

O estado crítico de saúde de um paciente sofre a interferência de múltiplos fatores. Diversos fatores externos e de fatores intrínsecos podem determinar simultaneamente a susceptibilidade e o desfecho a partir da condição crítica. Cada efeito externo e cada um dos genes herdados exercerão isoladamente um pequeno efeito, mas que cumulativamente definirá o desfecho. O estado de saúde, o prognóstico e o desfecho desses pacientes estão, portanto, muito relacionados à herança genética que o indivíduo recebeu. Muitos autores relacionaram a presença de alterações no *CD14*, *TLR4*, *TLR2* e *TNF-a* a uma resposta anormal do organismo a agentes infecciosos e a processos inflamatórios. O *CD14*, um receptor de reconhecimento padrão de moléculas envolvidas na resposta imune inata contra fatores exógenos e endógenos de estresse juntamente com os seus co-receptores mais importantes *TLR2*, *TLR4*, medeiam a resposta inflamatória por endotoxinas. Este processo é responsável por desencadear a produção do fator de necrose tumoral (*TNF-a*), que além de seu papel protetor na imunidade inata, pode exercer também efeitos patogênicos. O crescente aumento no número de mortes relacionadas a complicações decorrentes de processos inflamatórios, infecção, sepse e choque séptico em UTIs em todo país e no mundo (47.3% de óbitos no Brasil; 44,7% de óbitos na China; 36% de óbitos na União Européia e 34.7% de óbitos nos Estados Unidos), estimulou a busca e ao estudo para verificar se a herança destes receptores e de citocinas pró-inflamatórias poderiam influenciar no desfecho clinico de pacientes críticos internados no Hospital São Lucas da PUCRS (HSL). Em 2006 foi pesquisado a influencia do SNP -260C>T em 85 pacientes críticos internados no HSL, com a verificação de que os pacientes que apresentaram o genótipo -260TT tinham maior taxa de sobrevida do que os demais pacientes. Em 2010 foi testado uma amostra de 514 pacientes em estado crítico se o genótipo -260TT *CD14* ocorreria mais frequentemente entre os sobreviventes do que entre os pacientes falecidos. Este estudo publicado mostrou que os resultados de 2006 se confirmaram com uma maior robustez O SNP -260C>T *CD14* foi um fator protetor para a sobrevivência em pacientes gravemente doentes: houve uma frequência superior de sobreviventes entre os homozigotos -260TT *CD14*. Os resultados obtidos e a hipótese levantada, sugeriram que a maior freqüência do genótipo -260TT *CD14* em pacientes de UTI sobreviventes seria, possivelmente, explicada por um efeito de sinalização na imunidade inata. A literatura naquele momento (2009) sugeria que a análise de uma série de marcadores genéticos polimórficos poderia ser mais informativa do que a análise de um único polimorfismo. Então, o foco passou a ser a análise dos SNPs 896A>G e 1196C>T do *TLR4*; 2258G>A e 2029C>T do *TLR2* e o -308G>A do *TNF-a* que possuem ação sinérgica com o *CD14* para verificar se eles também poderiam ser informativos no desfecho dos pacientes com condições críticas de saúde. Os resultados, então, mostraram que SNPs nos genes *TLR2*, *TLR4* e *TNF-a*, isoladamente, não desempenham um papel significantemente notável no desfecho da doença crítica. No entanto, quando foi realizada uma análise combinada com a herança do -260C>T *CD14*, foi detectada uma taxa de sobrevivência significativamente maior no grupo de pacientes duplo homozigoto -260TT *CD14*/-308GG *TNF-*

α. Na análise ajustada com o duplo genótipo e as principais variáveis clínicas preditoras de mortalidade (SOFA, Idade, APACHE II, Tempo de UTI, Sepse/Choque séptico), foi observado que o duplo genótipo -260TT *CD14/*-308GG TNF- α foi um fator importante de proteção para a sobrevivência. Coeso ao efeito benéfico do -260TT *CD14*, o genótipo -308GG *TNF-α* foi protetor contra a relatada superexpressão de *TNF-α* causada por alelo *-308A TNF-α*. Estudos sobre infecção, sepse, choque séptico e mortalidade com pacientes críticos tem buscado ao longo dos anos, alternativas para tratamentos com custos não elevados e com um percentual satisfatório de recuperação destes pacientes. Em 2003 segundo dados do Instituto latino americano de sepse, foram gastos com o tratamento de pacientes críticos, um total de 17,34 bilhões de reais. Até a presente data, é possível observar que os estudos sobre o assunto têm levantado a possibilidade de que determinados genes possam influenciar no desfecho de pacientes. Apesar destes resultados positivos, acredita-se estar longe a obtenção de uma resposta concreta sobre quais os reais fatores que desencadeiam uma resposta inflamatória sistêmica. Há ainda muito que se descobrir sobre a resposta inflamatória e de como o organismo humano responde a estes estímulos, e por que algumas pessoas tendem a ter uma resposta exacerbada, mesmo não sendo encontrada associação estatística com os principais sinalizadores de inflamação. Trabalhar com estas seis variantes polimórficas (896A>G *TLR4,* 1196C>T *TLR4,* 2029C>T *TLR2,* 2258G>A *TLR2,* -308G>A *TNF-α e* -260C>T *CD14*) de diferentes genes responsáveis pela resposta inflamatória foi um esforço na busca de desvendar parte do pequeno efeito que a herança genética pode ter sobre o processo inflamatório durante a condição crítica e sua relação com o desfecho clínico.

As conclusões foram que:

1- Mesmo isoladamente, o SNP -260C>T *CD14* foi capaz de se mostrar um fator significativamente protetor para a sobrevivência em pacientes gravemente doentes: houve uma frequência superior de sobreviventes homozigotos -260TT *CD14*.

2- Não foi possível observar efeito significativamente diferente no desfecho da doença crítica entre a herança das variantes alélicas *896G TLR4*, *1196T TLR4*, *2029T TLR2*, *2258A TLR2* e *-308A TNF-α*.

3- A análise combinada da herança do duplo genótipo -260TT *CD14/*-308GG *TNF-α* foi um fator significativamente importante de proteção para a sobrevivência podendo influenciar o desfecho de pacientes criticamente enfermos.

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