MARIA LILIAN SALES

IMPACTO DOS POLIMORFISMOS PRO249SER DO TOLL-LIKE RECEPTOR-6 E ASP299GLY DO TOLL-LIKE RECEPTOR-4 SOBRE A ESTRUTURA VENTRICULAR ESQUERDA EM PACIENTES HIPERTENSOS

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Tese de Doutorado apresentada à Comissão de Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do título de Doutor em Clínica Médica.

Orientador: *Prof. Dr. Wilson Nadruz Júnior*

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Prof. Dr. Wilson Nadruz Júnior
Prof. Dr. Raimundo Marques Nascimento Neto
Prof. Dr. Leonardo Antônio Mamede Zornoff
Prof. Dr. Luiz Antonio Kannebley
Prof. Dr. Lício Augusto Velloso

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Banca examinadora da tese de Doutorado

Maria Lílian Sales

Orientador: Prof. Dr. Wilson Nadruz Júnior

Membros:

1. Prof. Dr. Raimundo Marques Nascimento Neto
2. Prof. Dr. Leonardo Antônio Mamede Zornoff
3. Prof. Dr. Licio Augusto Velloso
4. Prof. Dr. Luiz Antonio Kannebley Bittencourt
5. Prof. Dr. Wilson Nadruz Júnior

Curso de pós-graduação em Clínica Médica da Faculdade de Ciências Médicas da Universidade Estadual de Campinas.

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Dedico este trabalho...

À minha mãe, ao meu pai e ao meu irmão, por serem minha base e minha direção.

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“Se as coisas são
inatingíveis... ora!
Não é motivo para não
querê-las...
Que tristes os caminhos, se
não fora
A presença distante das
estrelas!

Mário Quintana

RESUMO

Estudos experimentais revelaram que a inibição de componentes da via de sinalização celular regulada pelos Toll-Like Receptors (TLRs) pode atenuar a hipertrofia cardíaca em animais submetidos à sobrecarga pressora. O objetivo deste estudo foi investigar a influência dos polimorfismos Asp299Gly do TLR4 e Pro249Ser do TLR6 sobre a estrutura do ventrículo esquerdo (VE) em indivíduos hipertensos. Foram estudados 443 pacientes por meio de avaliação clínica, laboratorial e ecocardiográfica, enquanto que os polimorfismos foram detectados por reação de polimerase em cadeia/enzima de restrição. Ademais, monócitos obtidos de sangue periférico de pacientes hipertensos foram estimulados *in vitro* com LPS (agonista de TLR4) e zimosan (agonista de TLR6) e a produção de interleucina-6 e fator de necrose tumoral-alfa (TNF-alfa) foi avaliada de acordo com a presença da variante Asp299Gly do TLR4 e Pro249Ser do TLR6. Mulheres que carregavam o alelo TLR4 299Gly apresentaram menor espessura de parede posterior do VE, menor espessura de septo, menor índice de massa do VE e reduzida prevalência de hipertrofia cardíaca. Mulheres homozigotas para TLR6 249Ser apresentaram menor espessura da parede do VE e menor espessura relativa da parede do VE em relação às mulheres com os genótipos Pro/Ser e Pro/Pro. Estes achados foram confirmados por análise de regressão linear múltipla, que incluiu idade, pressão arterial sistólica e diastólica, índice de massa corpórea, menopausa, diabetes mellitus e uso de anti-hipertensivos como fatores confundidores. Por fim, estudos funcionais *in vitro* revelaram que monócitos de homens e mulheres que apresentaram o alelo TLR4

299Gly tiveram menor produção de interleucina-6 após estímulo com LPS e a presença do alelo TLR6 249Ser em homozigose esteve associada a uma menor produção de interleucina-6 e TNF-alfa após estímulo com zimosan apenas nos monócitos extraídos de mulheres hipertensas. De maneira geral, esses dados sugerem que pode haver uma interação entre gêneros, polimorfismos dos receptores Toll-like e fenótipo do VE em indivíduos hipertensos. Sob esta perspectiva, estudos longitudinais são necessários para avaliar o impacto destes polimorfismos sobre o risco cardiovascular em mulheres hipertensas.

ABSTRACT

Experimental studies have shown that inhibition of components of cell signaling pathway regulated by Toll-Like Receptors (TLRs) can reduce cardiac hypertrophy in animals subjected to pressure overload. The aim of this study was to investigate the influence of the polymorphisms TLR4 Asp299Gly and TLR6 Pro249Ser on the structure of the left ventricle in hypertensive subjects. We studied 443 patients by clinical, laboratory and echocardiographic, while polymorphisms were detected by the polymerase chain / restriction enzyme. Moreover, monocytes obtained from peripheral blood of hypertensive patients were stimulated in vitro with LPS (TLR4 agonist) and zymosan (TLR6 agonist) and the production of interleukin-6 and tumor necrosis factor-alpha (TNF-alpha) was assessed according to the presence of the Asp299Gly variant of TLR4 and Pro249Ser of TLR6. Women who carried the TLR4 299Gly allele had lower posterior wall thickness, thinner septum, a lower rate of left ventricular mass and reduced prevalence of cardiac hypertrophy. Women homozygous for TLR6 249Ser had lower posterior wall thickness and relative wall thickness, compared to women with the genotype Pro/Ser and Pro/Pro. These findings were confirmed by analysis of multiple linear regression that included age, systolic and diastolic blood pressure, body mass index, menopause, diabetes mellitus and use of antihypertensive drugs as confounding factors. Finally, in vitro functional studies revealed that monocytes from men and women who harbored the TLR4 299Gly allele had lower production of interleukin-6 after stimulation with LPS and the presence of homozygous 249Ser allele was associated with a lower production of Interleukin-6 and TNF-alpha after stimulation with zymosan

only in monocytes hypertensive women. These data suggest that there may be an interaction between gender, polymorphisms of Toll-like receptors and ventricular phenotype in hypertensive subjects. In this regard, longitudinal studies are needed to assess the impact of these polymorphisms on the cardiovascular risk of hypertensive women.

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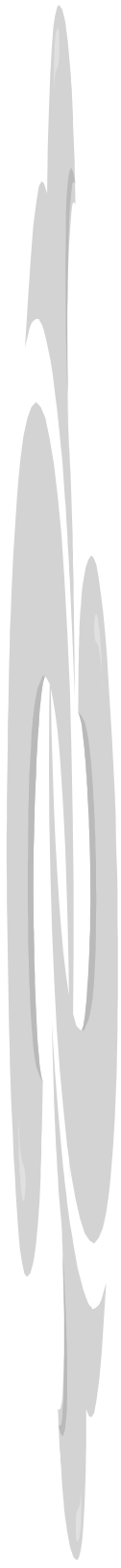

I. INTRODUÇÃO

I. INTRODUÇÃO

1.1. Hipertensão Arterial Sistêmica

A primeira mensuração da pressão arterial data de 1733, quando o reverendo anglicano Stephen Hales canula, de modo direto, a artéria de uma égua, iniciando, desta maneira, o conhecimento da quantificação da pressão arterial. Já em 1896, o italiano Scipione Riva-Rocci cria o esfigmomanômetro de coluna de mercúrio e, com este, afere a pressão arterial sistólica de modo indireto, não invasivo, pelo método oscilatório. Contudo, é apenas mediante a ausculta de sons desenvolvida por Nicolas Sergievich Korotkoff que em 1905 foi possível a quantificação da pressão arterial diastólica (1).

A aferição da pressão arterial é a terceira medida mais utilizada na prática médica, sendo apenas menos frequente que a medida da frequência de pulso e da temperatura. A medida da pressão arterial é o elemento-chave para o estabelecimento do diagnóstico da Hipertensão Arterial Sistêmica e avaliação da eficácia do tratamento. Considera-se hipertenso o indivíduo que apresenta duas ou mais medidas maiores ou iguais a 140/90 mmHg em ocasiões diferentes (2).

A elevação da pressão arterial representa um fator de risco independente, linear e contínuo para a morbi-mortalidade cardiovascular. A mortalidade cardiovascular aumenta progressivamente a partir de 115/75 mmHg (3). Entre os fatores de risco para a doença cardiovascular, a hipertensão arterial explica 40% das mortes por acidente vascular cerebral e 25% daquelas por doença coronariana (4).

De acordo com o critério de diagnóstico de hipertensão arterial (pressão

arterial ≥ 140/90 mmHg), a prevalência de hipertensão na população adulta brasileira varia de 22,3 a 43,9% (Figura 1).

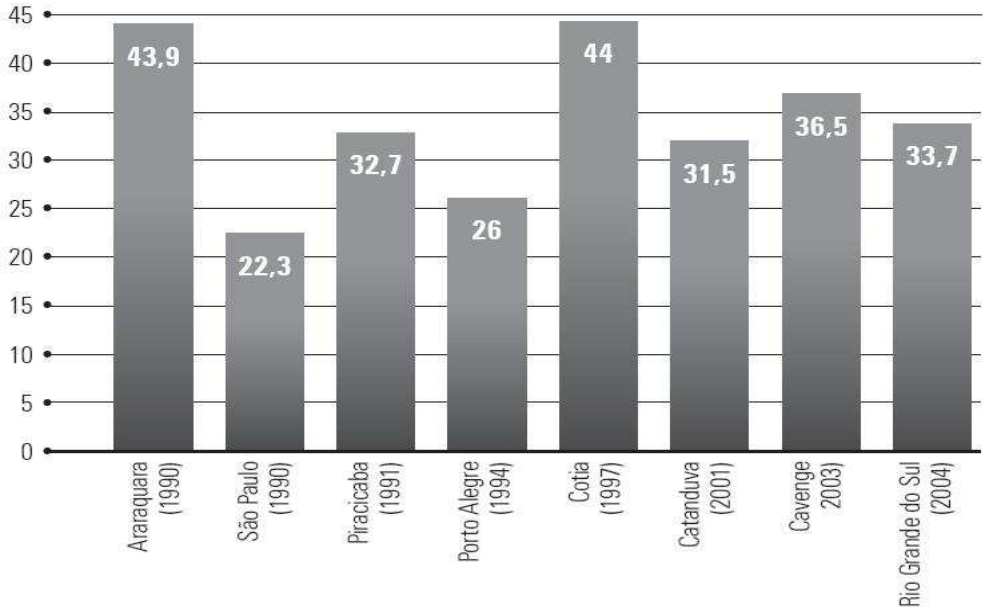

Figura 1: Prevalência de Hipertensão Arterial Sistêmica em cidades brasileiras. (V Diretrizes, 2007).

A hipertensão arterial, bem como as doenças a ela relacionadas são responsáveis por alta frequência de internações, sendo a insuficiência cardíaca a principal causa. O custo global de internações no Brasil por doença cardiovascular em 2005 foi de R$1.323.775.008,28 (2).

1.2. Hipertrofia de Ventrículo Esquerdo

A hipertensão arterial não é apenas a manifestação hemodinâmica de níveis pressóricos alterados, mas o prenúncio de alterações estruturais e funcionais complexas dos órgãos-alvo (5).

A hipertrofia ventricular esquerda induzida pela hipertensão arterial é inicialmente um processo adaptativo em resposta à sobrecarga pressórica, contudo também representa um fator de risco para a doença cardiovascular, independente da elevação das pressões sistólica e diastólica (6). Em metanálise de 20 estudos que envolveram 48.000 pacientes, encontrou-se risco médio ponderado de morbidade cardiovascular associado à hipertrofia ventricular esquerda de 2,3 vezes (7).

Estima-se que para cada acréscimo de 20 mmHg na pressão arterial sistólica, o risco relativo para o desenvolvimento de hipertrofia do ventrículo esquerdo aumente em 43% entre homens e 25% entre mulheres (8).

A incidência de hipertrofia ventricular esquerda em pacientes com hipertensão arterial leve a moderada se estabelece entre 12 e 30% no caso de hipertensos em seguimento em unidades de atenção primária e entre 20 e 60% para os pacientes acompanhados em centros de referência (9).

O acompanhamento por três anos de 1033 hipertensos, com idade superior a 50 anos e sem doença cardiovascular no início do estudo, mostrou que eventos clínicos tais como infarto agudo do miocárdio, insuficiência cardíaca congestiva e mortalidade cardiovascular são muito mais frequentes naqueles com massa ventricular superior a 125g/m^2. Cada 39g/m^2 de aumento da massa ventricular foi associado a risco 40% maior desses eventos(10). O *The Cardiovascular Health Study*, que acompanhou por um período de cinco anos 3042 pacientes com índice de massa ventricular maior que a média revelou que a hipertrofia de ventrículo esquerdo foi fator preditor independente para a fibrilação atrial, ondas Q, diminuição da fração de ejeção ventricular esquerda e infarto do miocárdio (11).

As alterações ventriculares esquerdas podem ser divididas em três padrões

geométricos: remodelamento concêntrico (aumento da espessura relativa da parede ventricular, porém com massa cardíaca normal); hipertrofia concêntrica (aumento da espessura relativa da parede ventricular e da massa cardíaca) e hipertrofia excêntrica (aumento da massa cardíaca com elevação do volume da cavidade ventricular) (12). O padrão geométrico do ventrículo esquerdo pode influenciar o desenvolvimento de insuficiência cardíaca. Estudos mostraram que a hipertrofia concêntrica se apresenta como o padrão geométrico ventricular mais associado à progressão para essa condição (12). A definição da geometria do ventrículo esquerdo, em particular o remodelamento concêntrico, tem sido apontada como importante marcador de risco cardiovascular, mesmo em pacientes com massa ventricular esquerda normal (13).

Embora a sobrecarga pressórica seja o principal fator determinante para o remodelamento ventricular e a hipertrofia de ventrículo esquerdo, fatores como ingestão de sal, atividade simpática, níveis de neurohormônios, fatores autócrinos e parácrinos, etilismo, obesidade, *diabetes mellitus*, exercício físico e estresse oxidativo também podem contribuir para a variabilidade da massa cardíaca em hipertensos (14).

Alguns estudos têm sugerido também a influência de fatores genéticos no desenvolvimento da hipertrofia ventricular esquerda, inicialmente sugerida por observações epidemiológicas em filhos normotensos de pais hipertensos, que apresentam massa ventricular esquerda maior do que indivíduos com pais normotensos (15). Estima-se que 60% da variação da massa ventricular esquerda seja causada por fatores genéticos independentes da pressão arterial (16). A análise da massa ventricular esquerda por ecocardiografia no *Framingham Heart Study* indicou significativa correlação da massa de ventrículo esquerdo entre pais e filhos bem como entre irmãos após correção para idade, altura, peso e pressão arterial

sistólica (17, 18).

Diversos fatores podem estar envolvidos no desenvolvimento de hipertrofia cardíaca, tais como moléculas de sinalização celular, hormônios, fatores de crescimento, pressão arterial e mutações de genes que codificam proteínas sarcoméricas (14). Por outro lado, alguns estudos recentes têm demonstrado também a influência dos receptores *Toll-like* sobre o desenvolvimento da hipertrofia ventricular esquerda em modelos animais (19, 20).

1.3. Receptores Toll-like

Os receptores *Toll-like* são proteínas essenciais para a imunidade inata, reconhecendo padrões expressos em diversos microorganismos. Em *Drosophilas*, o receptor transmembrana *Toll* desempenha um papel central nas vias de sinalização da formação do eixo ventral-dorsal, como também na resposta imune inata não específica. O receptor *Toll-like* expresso em humanos é uma proteína transmembrana tipo I, com uma porção extracelular que consiste de uma região de repetição rica em leucina e uma intracelular homóloga ao receptor interleucina-1 (IL1R). A via de sinalização do *Toll* é paralela à via de sinalização induzida por IL1R em células de mamíferos, e ambas utilizam a via do *NF-kappa-B*. Assim, o sistema de resposta imunológica mediada por *Toll* representa uma família evolucionariamente conservada do sistema de defesa do hospedeiro (21). Os receptores *Toll-like* fazem parte de uma família de receptores do sistema de imunidade inata, denominada receptores de reconhecimento de padrões. Estima-se que o sistema imunológico inato possa reconhecer cerca de 10^3 padrões moleculares de microorganismo, sendo que cada

receptor reconhece padrões específicos.

O estudo que identificou sequências de DNA dos primeiros cinco receptores *Toll-like*, denominados TLRs 1 a 5, data de 1998. Pelo uso de *Northern Blot*, foram determinados os padrões de expressão de todos os cinco genes. Os TLRs contêm matriz extracelular rica em repetições de leucina e um domínio citoplasmático de sinalização (Figura 2). O domínio citoplasmático de homologia TIR é semelhante ao encontrado nas caudas citoplasmáticas dos receptores para citocina IL-1 e IL-18, sendo que vias semelhantes de sinalização são iniciadas pelos TLRs, IL-1 e IL-18. A estrutura prevista é topologicamente idêntica às estruturas de reguladores da resposta à infecção bacteriana do receptor *Toll* da mosca *Drosophila*, o que justifica a proposição de que essa proteína faz parte de uma primitiva via de imunidade inata semelhante em insetos, plantas e animais (22).

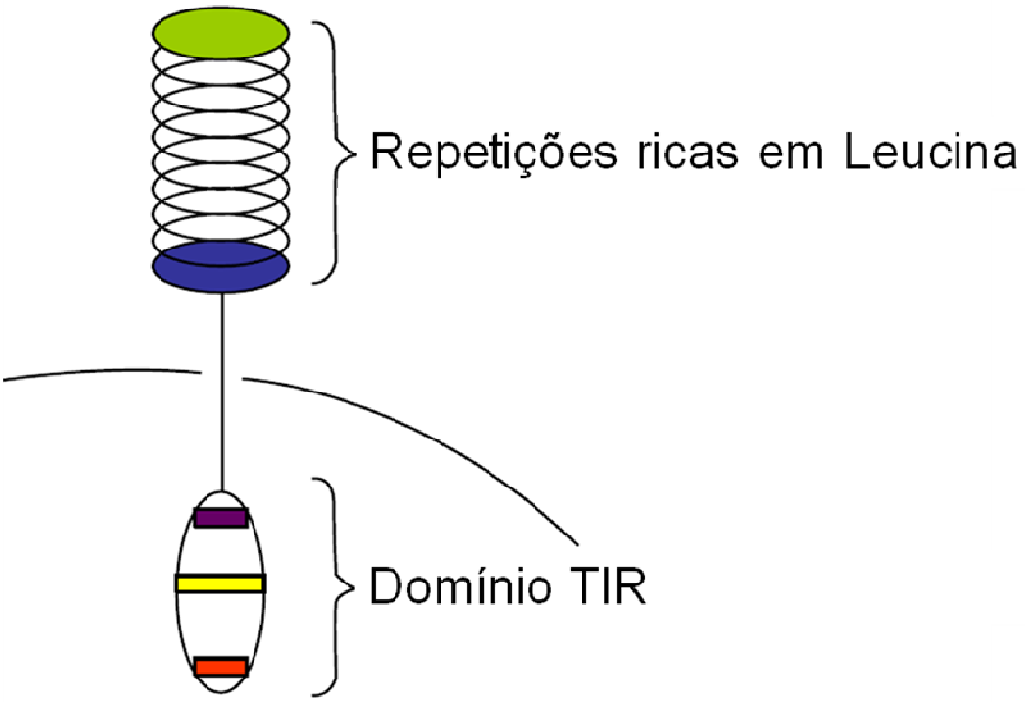

Figura 2: Estrutura Receptor Toll-like. Figura adaptada de Krishnan ET AL, 2007.(23).

São conhecidos 12 receptores Toll-like em mamíferos até o momento, e 11 em humanos. Esses receptores são expressos em diferentes tipos celulares, incluindo macrófagos, células dendríticas, neutrófilos, células epiteliais, células endoteliais, fibroblastos e cardiomiócitos (24). TLRs são encontrados tanto na superfície celular quanto em membranas intracelulares (endossomos), sendo capazes de reconhecer microorganismos em diferentes localizações celulares (Figura 3).

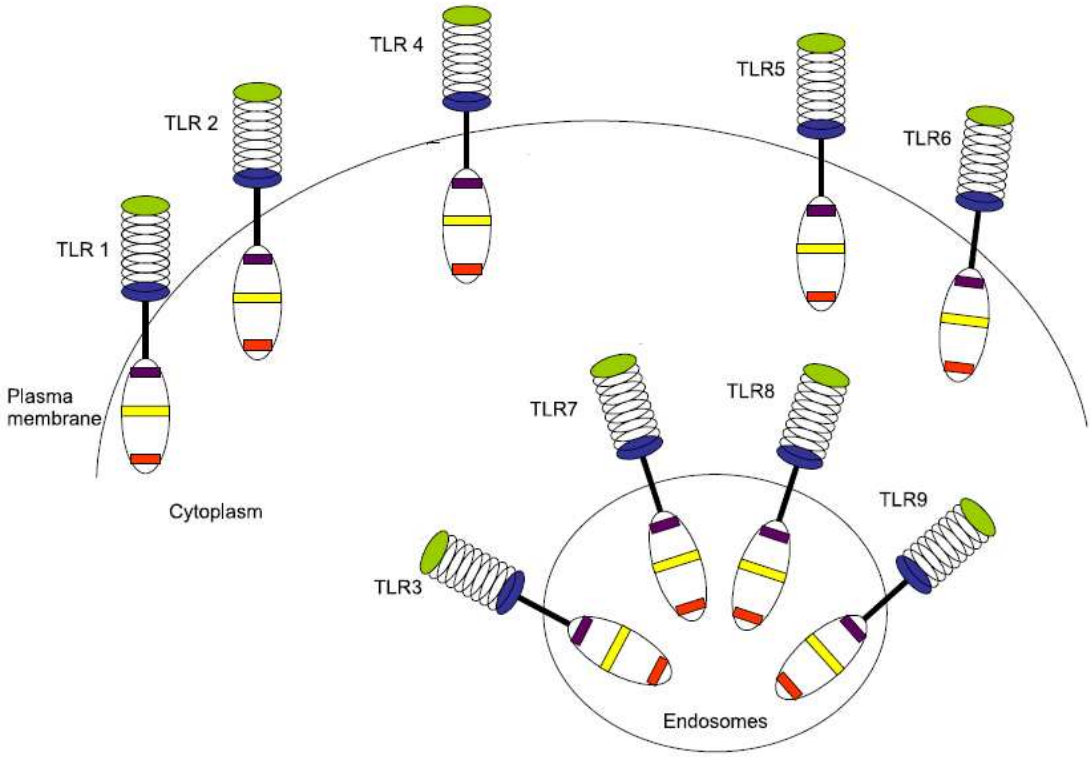

Figura 3: Localização Receptores Toll-like. Figura adaptada de Krishnan ET AL, 2007.(23).

Cada receptor da família *Toll-like* reconhece alguns padrões específicos que são comumente expressos em células microbianas, como mostrado na Tabela 1.

TLRs	Padrões reconhecidos
TLR 1	Lipopeptídeos bacterianos
TLR 2	Lipopeptídeos bacterianos, peptideoglicano, lipoproteína, ácido lipoteicóico
TLR 3	RNA bifilamentar viral
TLR 4	Lipopolissacarídeos bacterianos (LPS)
TLR 5	Flagelina bacteriana
TLR 6	Zymosan, lipopeptídeo, ácido lipoteicóico
TLR 7	RNA monofilamentar viral
TLR 8	RNA monofilamentar viral
TLR 9	CpG DNA não metilado
TLR 10	Desconhecido
TLR 11	Profilina

Tabela 1: Receptores Toll-like e ligantes específicos (padrões reconhecidos). Krishnan ET AL, 2007.

1.3.1. Vias de Sinalização dos TLR

A sinalização pelo TLR exige a dimerização das proteínas TLR na membrana celular, podendo ocorrer homodimerização de duas proteínas TLR idênticas ou heterodimerização de duas proteínas TLR diferentes. O repertório de especificidades do sistema TLR é ampliado pela capacidade dos receptores de se dimerizarem. Após a dimerização dos TLRs induzidas pelo ligante ocorre ligação de proteínas adaptadoras citoplasmáticas às caudas dos receptores *Toll-like*, via interação homotípicas de domínios encontrados tanto no TLR quanto na proteína adaptadora. São conhecidas quatro proteínas adaptadoras que interagem com os receptores da família *Toll-like*: MyD88 (fator de diferenciação mielóide 88), Mal (MyD88 semelhante

à adaptadora), TIRAP (proteína adaptadora contendo domínio TIR), Trif (adaptadora contendo domínio TIR induzindo interferon β). Diferentes combinações de adaptadoras são usadas por diferentes TLRs (23). Um importante efeito corrente abaixo da sinalização TLR é a ativação do fator de transcrição NF-κβ, essencial para a expressão de muitos genes relacionados com a imunidade natural e com a inflamação. Os passos básicos da sinalização por TLR são mostrados na Figura 4.

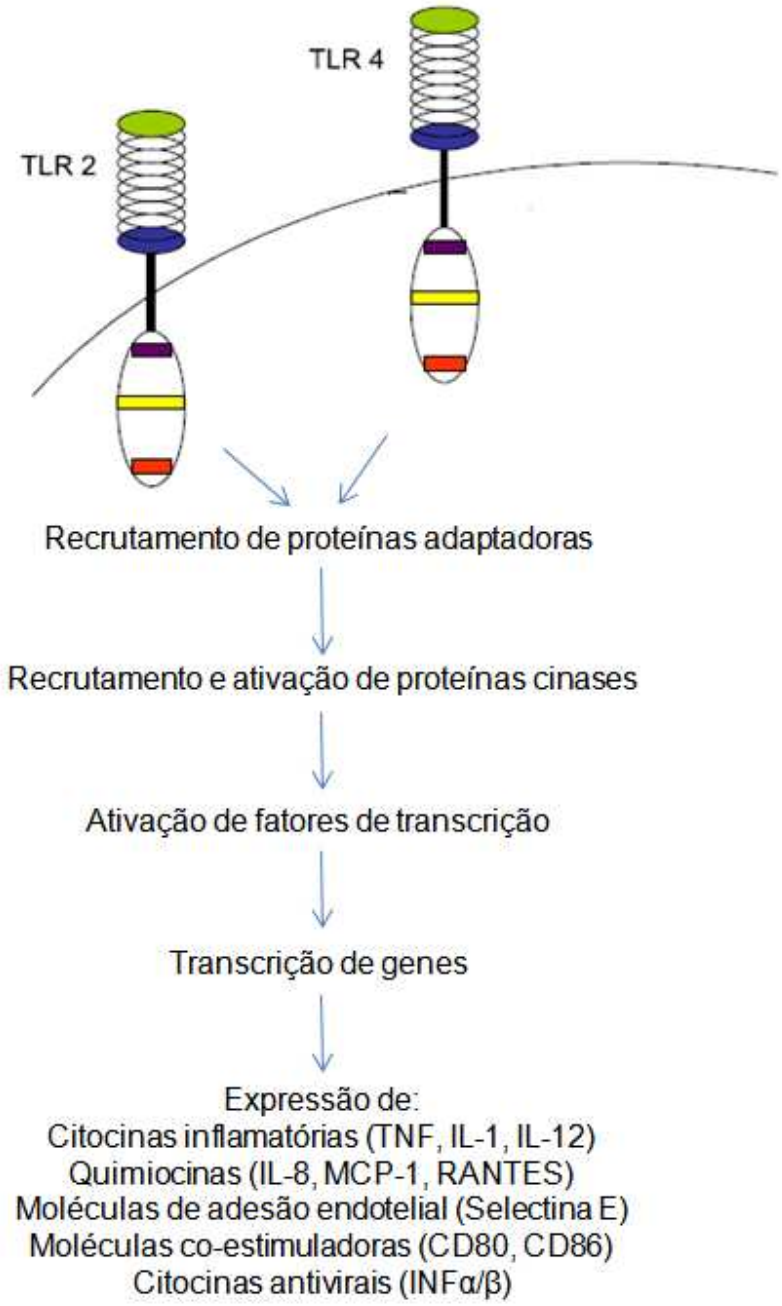

Figura 4: Passos básicos da via de sinalização dos Receptores Toll-like. Figura adaptada de Krishnan ET AL, 2007 (23).

Todos os receptores Toll-like se ligam à proteína adaptadora MyD88, exceto o

TLR 3. As vias de sinalização ativadas pelos TLRs podem, então, ser divididas em via dependente de MyD88 e via independente do MyD88. A via independente de MyD88 utiliza as proteínas adaptadoras Trif (proteína adaptadora contendo domínio TIR induzindo interferon-β) e TRAM (molécula adaptadora relacionada com a Trif) (23). Essa via pode ser ativada pelos TLRs 3 e 4 (25). As vias de sinalização dos TLRs são mostradas na Figura 5.

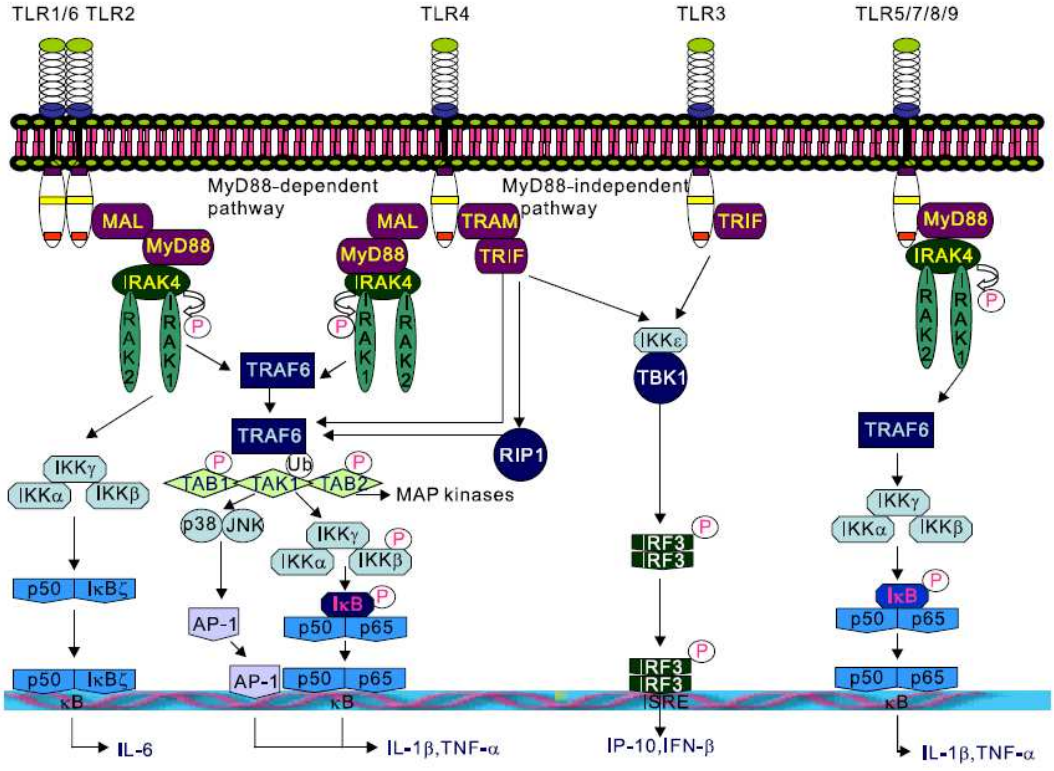

Figura 5: Vias de sinalização dos Receptores Toll-like (Dependente e independente do MyD88). Figura adaptada de Krishnan ET AL, 2007 (23).

1.4. Receptor Toll-like 4

O receptor *Toll-like* 4 é uma proteína transmembrana de 841 aminoácidos com características específicas dos receptores *Toll*, um domínio extracelular rico em repetições de leucina e uma região citoplasmática homóloga ao ILR1 (21). A expressão de TLR4 é alta em células isoladas de músculo cardíaco de ratos, murinos e miocárdio humano (26). Receptores do sistema imune reconhecem uma ampla variedade de moléculas. O TLR 4 reconhece o LPS bacteriano e inicia sua via de sinalização. Além do LPS, o TLR 4 pode ser ativado por fragmentos de tecido endógeno, como produtos da degeneração da matriz extracelular, fibrinogênio (27), fibronectina (28), certos tipos de proteínas do choque térmico (29) e lipoproteínas de baixa densidade (LDL) (30, 31). Quando o TLR 4 reconhece seus ligantes específicos, ativa uma via de resposta de imunidade inata (32).

Estudos têm mostrado que o TLR 4 pode estar envolvido com o desenvolvimento de hipertrofia ventricular. Em tecido cardíaco de pacientes com hipertrofia cardíaca idiopática foram encontradas áreas com intensa expressão de TLR 4 (26). Camundongos *knockout* para TLR 4 desenvolveram hipertrofia ventricular esquerda menos severa do que animais controle após bandagem de aorta (19), enquanto o bloqueio da proteína *MyD88*, um fator intermediário da via do TLR 4, também atenuou a hipertrofia cardíaca induzida por sobrecarga (20). Dados de estudo recente mostraram que o TLR4 influenciou o remodelamento do miocárdio pós infarto (33).

1.4.1. Polimorfismos TLR 4

Duas variantes do receptor *Toll-like* 4 são principalmente estudadas, sendo a

primeira a transição da base A para G na posição 896 que leva a troca de aminoácidos, de aspartato para glicina, polimorfismo chamado de asp299gly ou D299G (34, 35). Outra variante do TLR 4 é o thr399ile (T399I), no qual há uma troca na posição 399 do aminoácido treonina para leucina (35). Há evidências de que esses polimorfismos estão associados à menor resposta do receptor ao LPS. Modelos moleculares revelaram tanto que os aminoácidos localizados nas posições 299 e 399 situam-se na mesma face extracelular da proteína TLR 4, como também que a expressão dos aminoácidos dos polimorfismos altera a estrutura do receptor, o que pode mudar a interação do ligante com o receptor *Toll-like* 4 (36, 37).

1.4.2. Polimorfismos TLR4 e Doença Cardiovascular

A aterosclerose é caracterizada pela inflamação local crônica da parede vascular, que resulta em um acúmulo de lipídios e células derivadas de macrófagos no espaço subendotelial (38). Embora a natureza inflamatória do processo aterosclerótico tenha sido confirmada, os componentes da cascata que conduzem a esse processo permanecem desconhecidos. Estudos epidemiológicos têm sugerido uma ligação entre a ativação dos TLRs e a aterosclerose. Em 2002, um estudo encontrou a associação do polimorfismo Asp299Gly com reduzida aterosclerose e menor espessura de camada íntima-média carotídea (39), todavia, este resultado não foi encontrado posteriormente em estudo com mais participantes (40). Outros estudos tentam correlacionar o risco de infarto do miocárdio a polimorfismos do TLR4. O polimorfismo Asp299Gly foi associado a um menor risco de evento coronariano agudo (41) e, em outros dois estudos, foi associado ao menor risco de eventos

cardiovasculares em pacientes em uso de estatina (42, 43). Dois estudos posteriores não confirmaram este resultado (44). Os polimorfismos Asp299Gly e Thr399Ile foram associados ao aumento do risco de infarto em homens, mas não em mulheres (45).

Dados experimentais confirmam estes achados clínicos. Vários TLRs são expressos nas placas de aterosclerose pelas células locais ou pelos leucócitos que migraram para a parede arterial (30, 46). Ratos *knockout* para *MyD88* apresentam menos aterosclerose quando comparados a controles (47, 48). Em concordância com estas evidências, ratos hipercolesterolêmicos *knockout* para TLR4 também apresentaram significativa redução na carga aterosclerótica em comparação aos animais controle (48).

TLRs 2, 3, 4 e 6 são também expressos em cardiomiócitos (26, 49). A expressão de TLR 4 é aumentada no miocárdio dos pacientes com insuficiência cardíaca avançada (26, 50). Após sobrecarga pressórica causada por bandagem da aorta, ratos *knockout* para TLR 4 apresentaram hipertrofia cardíaca menos intensa do que os ratos controles (19). O bloqueio de MyD88 reduz significativamente a hipertrofia ventricular causada após bandagem da aorta, além de reduzir a apoptose de cardiomiócitos (37). Esses achados indicam que a via TLR possui um importante papel no desenvolvimento da hipertrofia cardíaca. Contudo, permanece desconhecido o papel de polimorfismo de TLRs sobre o remodelamento ventricular em pacientes hipertensos.

1.5. Receptor Toll-like 6

O receptor TLR6 foi descrito em 1999 quando Takeuchi *et. al.* isolaram o cDNA que codifica a proteína Toll 6 humana; por sua vez, essa é uma proteína transmembrana de 796 aminoácidos, apresentando 74% de semelhança com o TLR 6 de rato e 69% de similaridade com o TLR 1 humano. Este receptor contém uma porção extracelular rica em repetições de leucina e um domínio citoplasmático homólogo ao ILR 1, assim como os outros membros da família de receptores *Toll-like* humanos (51).

O receptor Toll-like 6 é ativado por lipopeptídeos diacilados, ácido lipoteicóico bacteriano e zimosan (Tabela 1). O zimosan é um polissacarideo da parede celular do fungo *Saccharomyces cerevisae* (52).

1.5.1. Polimorfismos TLR 6

Tantisira et. al. descreveram em seu estudo 53 polimorfismos do receptor *Toll-like* 6, 11 destes apresentando troca de aminoácidos. A variante mais estudada até o momento é a resultante da transição da base C para T na posição 744 que causa a troca de aminoácidos, de serina para prolina, polimorfismo chamado de ser249pro (53).

Este polimorfismo ser249pro foi associado com reduzido risco de asma e risco aumentado para aspergilose invasiva (53, 54). Até o momento, não existem estudos avaliando a relação do receptor TLR 6 ou seus polimorfismos com alterações

cardiovasculares.

Com base nestas informações introdutórias, a hipótese desse estudo é investigar se os polimorfismos Ser249Pro do TLR 6 e Asp299Gly do TLR 4 levam a redução da hipertrofia de ventrículo esquerdo em indivíduos hipertensos.

II. OBJETIVOS

II. OBJETIVOS

O objetivo deste estudo é investigar a influência dos polimorfismos Asp299Gly do receptor Toll-like 4 e Pro249Ser do receptor Toll-like 6 sobre a estrutura do ventrículo esquerdo em indivíduos hipertensos.

III. RESULTADOS

III. RESULTADOS

Os resultados da presente tese estão apresentados nos seguintes artigos:

"*The functional Toll-like receptor 4 Asp299Gly polymorphism is associated with lower left ventricular mass in hypertensive women*", aceito para publicação na *Clinica Chimica Acta.*

"*Toll-like receptor 6 Ser249Pro polymorphism is associated with lower left ventricular wall thickness and inflammatory response in hypertensive women*", aceito para publicação no *American Journal of Hypertension*.

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The functional Toll-like receptor 4 Asp299Gly polymorphism is associated with lower left ventricular mass in hypertensive women

Maria L. Sales [a,b,1], Roberto Schreiber [a,1], Maria C.S. Ferreira-Sae [a], Maruska N. Fernandes [a], Cristiane Piveta [a], José A.A. Cipolli [a], Antônio Calixto [a], José R. Matos-Souza [a], Bruno Geloneze [a], Kleber G. Franchini [a], Wilson Nadruz Jr. [a,*]

[a] Department of Internal Medicine, School of Medicine, University of Campinas, Brazil
[b] Department of Medical Sciences, Federal University of Ouro Preto, Brazil

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ABSTRACT

Background: This study investigated the impact of a putative functional TLR4 polymorphism (Asp299Gly) on left ventricular (LV) structure in hypertensive subjects.
Methods: A sample of 443 patients (266 women and 177 men) was evaluated by clinical history, physical examination, anthropometry, analysis of inflammatory and metabolic parameters, echocardiography and TLR4 Asp299Gly genotyping. In addition, the relationship between the polymorphism and *in vitro* lipopolysaccharide responsiveness of peripheral blood monocytic cells was also assessed.
Results: Women carrying the 299Gly allele presented lower posterior wall thickness ($p = 0.01$), interventricular septum thickness ($p = 0.04$), LV mass ($p = 0.01$) and LV mass index ($p = 0.03$), as well as a reduced prevalence of LV hypertrophy ($p = 0.002$), in comparison to women with the wild-type genotype. These results were confirmed by stepwise and logistic regression analyses adjusted for potential confounders. Conversely, the 299Gly allele did not influence LV structure in men. Furthermore, *in vitro* assays revealed that monocytes of either men or women heterozygous for the 299Gly allele presented a lower lipopolysaccharide-induced production of interleukin-6, compared to non-carriers.
Conclusions: The functional TLR4 Asp299Gly polymorphism is associated with lower LV mass in hypertensive women. These findings suggest that interactions among gender, LV remodeling and TLR4 gene variants may occur in hypertensive subjects.

1. Introduction

Toll-like receptors (TLRs) are an important link between innate immunity and a variety of clinical disorders, including cardiovascular diseases [1,2]. Experimental studies have shown that TLRs might be involved in the development of left ventricular (LV) hypertrophy induced by pressure overload. In this regard, TLR4 knockout mice developed less-severe LV hypertrophy following aortic banding than matched wild-type animals [3], while blockade of MyD88, an intermediate factor of TLR4 pathway, also attenuated load-induced cardiac growth [4]. Moreover, recent data revealed that TLR4 mediates maladaptive LV remodeling in mice subjected to myocardial infarction [5], strengthening the notion that this molecule is a key regulator of cardiac structure following myocardial injury.

The TLR4 Asp299Gly polymorphism is associated with a blunted receptor activity and a subsequently diminished inflammatory response in humans [6–9]. This variant has been related to lower carotid intima–media thickness, increased arterial elasticity and diminished prevalence of myocardial infarction in some populations [10–13]. Nevertheless, little is known about the impact of TLR polymorphisms on cardiac phenotype. Thus, the aim of the present report was to investigate whether the TLR4 Asp299Gly variation was associated with alterations in LV structure in a sample of hypertensive subjects.

2. Materials and methods

2.1. Subjects

The study was carried out in 443 unrelated hypertensive subjects, comprising 266 women and 177 men, followed up at the Hypertension Unit of a university hospital. Hypertension was defined as systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg (systolic blood pressure ≥130 mm Hg or diastolic blood pressure ≥80 mm Hg for diabetics) or current antihypertensive medication

* Corresponding author. Departamento de Clínica Médica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Cidade Universitária Zeferino Vaz, 13081-970 Campinas, SP. Brazil. Tel./fax: +55 19 3521 7836.
E-mail address: wilnj@fcm.unicamp.br (W. Nadruz).
[1] Both authors contributed equally to this study.

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use. Diabetes mellitus was diagnosed if fasting blood glucose was ≥126 mg/dl on 2 separate tests or when participants were taking hypoglycaemic medications [14]. Women with reported amenorrhea for >12 months, except for pregnancy, were identified as postmenopausal. Coronary heart disease was diagnosed by history of myocardial infarction, acute coronary syndrome or coronary revascularization or by evidence of cardiac ischemia documented by functional testing. Main exclusion criteria were age under 18 years, significant cardiac valve disease, hypertrophic cardiomyopathy and neoplastic disease. The study was approved by the Ethics Committee of our institution and informed consent was obtained from all participants.

Blood pressure was measured using a validated digital oscillometric device (HEM-705CP; Omron Healthcare, Kyoto, Japan) with appropriate cuff sizes. Two readings were averaged and, if they differed by >5 mm Hg, one additional measurement was performed and the average of the three measurements was taken.

Body mass index was calculated as body weight divided by height squared (kg/m^2), while waist circumference was measured at the midpoint between the lowest rib and iliac crest. Fasting blood total cholesterol, low-density-lipoprotein cholesterol, high-density-lipoprotein cholesterol, triglycerides, uric acid, glucose, insulin and C-reactive protein levels were measured using standard laboratory techniques. The homeostasis model assessment index (HOMA) was calculated as: glucose (mg/dl) × insulin (μU/ml)/405 [15]. In addition, albumin/creatinine ratio in morning urine samples and creatinine clearance were also measured.

2.2. Echocardiography

Echocardiography studies were performed by a skilled physician using a Vivid 3 Pro (General Electric, Milwaukee, USA) apparatus equipped with a 2.5 MHz transducer as previously described [16]. LV end-diastolic and end-systolic diameters, interventricular septum thickness, posterior wall thickness and LV mass were measured in accordance with the American Society of Echocardiography guidelines [17]. Relative wall thickness was computed as twice the posterior wall thickness divided by LV end-diastolic diameter. LV mass index was considered as LV mass/$height^{2.7}$ and LV hypertrophy was defined with the use of a cutoff point >51 $g/m^{2.7}$ [18]. All the recordings were made by the same physician, who was unaware of other data relating to the subjects. The reproducibility of both acquiring and measuring LV mass was determined in recordings obtained from 10 subjects. The results found for intraobserver and interobserver variabilities were compared by using Bland–Altman analysis and were presented as the mean difference between the measurements (bias) ± 95% limits of agreement. The mean differences in LV mass were −3.41 ± 11.96 g for intraobserver measurements and +4.78 ± 12.72 g for interobserver measurements.

2.3. Genotyping

The Asp299Gly polymorphism was analyzed by polymerase chain reaction and digestion with NcoI restriction enzyme [9]. Genomic DNA was extracted from peripheral blood leucocytes. The polymerase chain reaction mixture contained 1 μl 10× polymerase chain reaction buffer, 2 mmol/l $MgCl_2$, 0.2 mmol/l dNTP mix, 1 μmol/l of both primers, 1 U recombinant Taq DNA Polymerase (Invitrogen, Eugene OR) and 100 ng genomic DNA in a total reaction volume of 10 μl. Primers for TLR4 Asp299Gly were F5′-GATTAGCATACTTAGACTACTACCTCCATG-3′ (forward) and R5′-GATCAACTTCTGAAAAAGCATTCCCAC-3′ (reverse). The polymerase chain reaction conditions were as follows: initial denaturation at 95 °C for 15 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 40 s and extension at 72 °C for 30 s, ending with a final extension at 72 °C for 10 min and cooling to 15 °C in an Mastercycler epGradienteS Thermal Cycler (Eppendorf, Foster City, CA). The polymerase chain reaction products were digested with NcoI restriction enzyme (Fermentas International Inc., Burlington, Canada) at 37 °C overnight, producing fragments of 263 base pairs (bp) (Asp allele) or 222 + 41 bp (Gly allele). The cleavage products were electrophoresed on 2% agarose gel and stained with SYBR safe DNA gel stain (Invitrogen). The restriction fragments were detected by ultraviolet illumination.

2.4. Cell culture and in vitro assays

In vitro stimulation of monocytic cells was performed as previously described [19], with minor modifications. Briefly, peripheral blood monocytic cells from 28 hypertensive patients of different genotypes (20 wild-type and 8 heterozygous for the TLR4 polymorphism) were isolated by density gradient centrifugation over Histopaque 1077 and washed twice with RPMI 1640. Cells were resuspended at 2×10^6 cells/ml and incubated in 1 ml of RPMI containing 10% fetal calf serum in 24-well tissue culture plates. Monocytes were isolated by adherence for 2 h in a humidified 5% CO_2 incubator at 37 °C; nonadherent cells were removed by changing the medium. Monocytes were then stimulated with 10 μg/ml lipopolysaccharide (Sigma Corporation, St. Louis, MO) for 24 hours and interleukin-6 and tumor necrosis factor-α in the supernatants were determined by ELISA (R&D Systems, Minneapolis MN).

2.5. Statistical analysis

Descriptive statistical results are given as means ± SEM. χ^2 and unpaired *t*-tests were used to compare categorical and continuous variables, respectively. Stepwise multiple regression analysis evaluated the independent predictors of continuous echocardiographic parameters, while logistic regression analysis assessed the predictors of LV hypertrophy. Variables included in multivariate models showed no multicollinearity. Differences between the mean values of interleukin-6 and tumor necrosis factor-α within each gender were evaluated by one-way ANOVA followed by the Tukey test for pairwise comparisons. A $p<0.05$ was considered significant.

3. Results

The frequency of TLR4 Asp299Gly genotypes is presented in Table 1. χ^2 analyses revealed that the polymorphism distribution was not in Hardy–Weinberg equilibrium in the studied patients. Analyses split by gender showed that deviation from Hardy–Weinberg equilibrium was detected solely in women, but not in men.

The clinical and laboratory characteristics of hypertensive subjects are shown in Table 2. In order to enhance statistical power and given the described functional effect of the 299Gly allele in heterozygous individuals [8–10], Asp/Gly subjects were added to Gly/Gly ones. No differences regarding the clinical and laboratory features were detected in hypertensive men according to the genotype groups, except for lower log C-reactive protein levels in 299Gly carriers. Conversely, female 299Gly carriers exhibited lower log urinary albumin/creatinine ratio levels, prevalence of diabetes mellitus and use of calcium channel blockers and angiotensin-converting enzyme

Table 1
Genotype frequencies of TLR4 Asp299Gly polymorphism.

Genotype	Men ($n=177$)	Women ($n=266$)	Total ($n=443$)
Asp/Asp, *n* (%)	160 (90.4)	250 (93.9)	410 (92.6)
Asp/Gly, *n* (%)	16 (9.0)	14 (5.3)	30 (7.3)
Gly/Gly, *n* (%)	1 (0.6)	2 (0.8)	3 (0.7)
Asp allele	0.949	0.966	0.959
Gly allele	0.051	0.034	0.041

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Table 2
Clinical characteristics of hypertensive patients according to TLR4 Asp299Gly polymorphism.

Characteristics	Men		Women	
Genotype	Asp/Asp	Asp/Gly + Gly/Gly	Asp/Asp	Asp/Gly + Gly/Gly
N	160	17	250	16
Age, years	58.3 ± 1.0	58.3 ± 4.1	55.9 ± 0.8	56.0 ± 2.9
Body mass index, kg/m^2	30.2 ± 0.4	31.8 ± 1.6	31.9 ± 0.4	30.8 ± 1.3
Waist, cm	103.4 ± 1.0	109.5 ± 4.3	101.0 ± 1.0	95.4 ± 3.4
Systolic blood pressure, mm Hg	147.3 ± 2.0	149.1 ± 5.9	150.3 ± 1.5	148.2 ± 7.9
Diastolic blood pressure, mm Hg	85.5 ± 1.2	87.8 ± 3.4	87.4 ± 0.8	88.6 ± 2.9
Postmenopause, *n* (%)	–	–	180 (72)	12 (75)
Diabetes mellitus, *n* (%)	43 (27)	5 (29)	73 (29)	1 (6)†
Smokers, *n* (%)	25 (16)	0 (0)	17 (7)	1 (6)
Coronary heart disease, *n* (%)	43 (27)	4 (24)	25 (10)	0 (0)
LDL-cholesterol, mg/dl	108.7 ± 3.3	97.7 ± 10.2	115.0 ± 2.2	112.4 ± 10.2
HDL-cholesterol, mg/dl	47.0 ± 1.1	44.5 ± 2.4	54.5 ± 1.0	61.2 ± 6.6
Triglycerides, mg/dl	168.1 ± 9.3	158.9 ± 22.1	150.3 ± 5.2	124.2 ± 13.5
HOMA	3.9 ± 0.4	4.5 ± 1.0	4.6 ± 0.5	3.9 ± 0.8
Uric acid, mg/dl	6.6 ± 0.1	5.8 ± 0.5	5.5 ± 0.1	5.5 ± 0.4
Log C-reactive protein, mg/dl	−0.52 ± 0.04	−0.80 ± 0.14*	−0.45 ± 0.03	−0.45 ± 0.10
Log albumin/creatinine ratio, g/g	−0.84 ± 0.05	−0.76 ± 0.17	−0.77 ± 0.04	−1.08 ± 0.10†
Creatinine clearance rate, ml/min	86.8 ± 3.0	103.1 ± 9.8	88.7 ± 2.4	99.4 ± 5.0
Diuretics, *n* (%)	130 (81)	15 (88)	203 (81)	13 (81)
Calcium channel blockers, *n* (%)	83 (52)	11 (65)	127 (51)	4 (25)†
Beta-blockers, *n* (%)	74 (46)	7 (41)	120 (48)	7 (44)
ACEI or ARB, *n* (%)	139 (87)	15 (88)	208 (83)	10 (68)†

Legend: LDL — low-density lipoprotein; HDL — high-density lipoprotein; HOMA — homeostasis model assessment index; ACEI or ARB — angiotensin-converting enzyme inhibitors or angiotensin receptor blockers; *$p = 0.02$ and $^{\dagger}p = 0.04$ compared to sex-matched subjects.

inhibitors/angiotensin receptor blockers, compared to women with the wild-type genotype.

Echocardiographic data are demonstrated in Table 3. Women carrying the 299Gly allele presented lower posterior wall thickness, interventricular septum thickness, LV mass and LV mass index and a reduced prevalence of LV hypertrophy compared to non-carriers. Conversely, no significant association between the TLR4 variant and echocardiographic features was detected in hypertensive men.

Considering that the relationship between the TLR4 polymorphism and cardiac structure in hypertensive women could be influenced by potential confounders, stepwise and logistic regression analyses were performed (Tables 4 and 5). The 299Gly allele was independently related to posterior wall thickness, LV mass and LV mass index in models that included age, body mass index, diabetes mellitus, blood pressure, menopause status and use of antihypertensive medications as independent variables (Table 4). Accordingly, the polymorphism also emerged as an independent predictor of LV hypertrophy (Table 5). On the other hand, log urinary albumin/creatinine was significantly associated with diabetes mellitus ($R^2 = 0.097$; $p < 0.0001$), systolic blood pressure ($R^2 = 0.043$; $p = 0.0005$) and age ($R^2 = 0.014$; $p = 0.04$), but not with body mass index, diastolic blood pressure, menopause status, use of antihypertensive medications and the TLR4 Asp299Gly variant.

Given that the 299Gly allele associated with changes in LV mass solely in hypertensive women, we then evaluated whether there were gender-related differences in the inflammatory response of isolated monocytes from wild-type (Asp/Asp) and heterozygous (Asp/Gly) hypertensive patients. In order to address this issue, peripheral blood monocytes were challenged *in vitro* with a TLR4 agonist (lipopolysaccharide) and the expressions of interleukin-6 and tumor necrosis factor-α were evaluated in the supernatant (Fig. 1). Stimulated monocytes from heterozygous men and women exhibited a lower expression of interleukin-6, in comparison to sex-matched wild-type subjects. Conversely, no difference in tumor necrosis factor-α induction was observed between wild-type and heterozygous individuals of both genders.

4. Discussion

Experimental evidence showed that TLR4 signaling pathway plays a major role in LV hypertrophy induced by pressure overload [3,4]. In the present report we found that the TLR4 Asp299Gly variant was associated with lower LV wall thickness and LV mass in hypertensive women. Accordingly, results of logistic regression analysis revealed an independent association between the 299Gly allele and decreased prevalence of LV hypertrophy in females. On the other hand, *in vitro* assays demonstrated that peripheral blood monocytes of men and women heterozygous for the 299Gly allele presented a lower lipopolysaccharide-induced production of interleukin-6, compared to non-carriers. Taken together, these findings indicate that a functional TLR4 polymorphism is associated with variation in LV

Table 3
Echocardiographic features of hypertensive patients according to TLR4 Asp299Gly polymorphism.

Characteristics	Men		*p*	Women		*p*
Genotype	Asp/Asp	Asp/Gly + Gly/Gly		Asp/Asp	Asp/Gly + Gly/Gly	
N	160	17		250	16	
Interventricular septum thickness, mm	11.2 ± 0.1	11.2 ± 0.4	NS	10.7 ± 0.1	9.8 ± 0.4	0.04
Posterior wall thickness, mm	11.1 ± 0.1	11.1 ± 0.5	NS	10.6 ± 0.1	9.6 ± 0.3	0.01
LV end-diastolic diameter, mm	53.5 ± 0.6	53.1 ± 1.7	NS	48.6 ± 0.3	46.9 ± 1.2	NS
Relative wall thickness	0.42 ± 0.01	0.42 ± 0.02	NS	0.44 ± 0.01	0.41 ± 0.02	NS
LV mass (g)	305 ± 13	298 ± 28	NS	243 ± 5	195 ± 14	0.01
LV mass index, g/m$^{2.7}$	76.0 ± 1.8	76.2 ± 7.7	NS	74.8 ± 1.6	61.1 ± 5.1	0.03
LV hypertrophy, *n* (%)	145 (91)	14 (82)	NS	215 (86)	9 (56)	0.002

Legend: LV — left ventricular; NS — non-significant.

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Table 4
Stepwise regression analyses for echocardiographic parameters in hypertensive women.

Step	Variable	R^2 change	F ratio	p
Dependent: posterior wall thickness				
1	Body mass index	0.200	65.8	<0.00001
2	Systolic blood pressure	0.063	23.9	<0.00001
3	Diastolic blood pressure	0.014	5.2	0.02
4	TLR4 Asp299Gly polymorphism	0.013	4.8	0.03
5	Age	0.012	4.3	0.04
Dependent: LV mass				
1	Body mass index	0.232	79.5	<0.00001
2	Systolic blood pressure	0.039	14.1	0.0002
3	TLR4 Asp299Gly polymorphism	0.015	5.3	0.02
4	Age	0.012	4.6	0.03
5	Diastolic blood pressure	0.011	4.2	0.04
Dependent: LV mass index ($g/h^{2.7}$)				
1	Body mass index	0.204	67.3	<0.00001
2	Systolic blood pressure	0.050	17.5	0.00004
3	Age	0.029	10.6	0.001
4	TLR4 Asp299Gly polymorphism	0.012	4.4	0.04

Legend: All models included age, body mass index, systolic blood pressure, diastolic blood pressure, TLR4 Asp299Gly polymorphism, diabetes mellitus, menopause status, diuretics use, calcium channel blockers use, beta-blockers use and angiotensin-converting enzyme inhibitors/angiotensin receptor blockers use as independent variables. Only variables with significant association were presented. LV — left ventricular.

phenotype solely in hypertensive subjects, further suggesting that interactions among gender, LV structure and TLRs may occur.

Human data demonstrated sex-related differences in LV structure and remodeling that are particularly manifested in pressure overload states [20,21]. These discrepancies have been partially explained by variations in sexual hormone profile [21], body mass composition [15,22] and metabolic factors [23]. Emerging data have suggested that inherited traits might also play a role in this regard. For instance, polymorphisms of the estrogen receptor β gene were associated with LV mass and wall thickness solely in women [24], while variation at the androgen receptor gene showed significant association with LV hypertrophy in males with hypertrophic cardiomyopathy [25]. Our study provided novel evidence that TLR4 Asp299Gly polymorphism is associated with lower LV mass exclusively in hypertensive women. This finding is in agreement with recent experimental and clinical data demonstrating that TLRs might exert gender-distinct effects on non-cardiovascular diseases [26,27]. Interestingly, our results of *in vitro* assays revealed a lower interleukin-6 response to lipopolysaccharide in monocytes from hypertensive subjects heterozygous for the 299Gly allele of both genders, suggesting that this variation in *TLR4* might act in concert with gender-related acquired or genetic features to influence the complex determination of LV phenotype in hypertensive subjects. An intuitive explanation to the present findings would be the differences in sexual hormone milieu. In this regard, it is possible that chronic estrogen exposition interacted with TLR4 299Gly allele, thus modulating LV mass in hypertensive women. Nevertheless, the results of multivariate analyses showing no influence of

Table 5
Covariates of left ventricular hypertrophy identified by logistic regression analysis in hypertensive women.

Variable	Exp(B) (95% C.I.)	p
Body mass index (≥30 g/m^2)	5.05 (2.29–11.73)	<0.0001
TLR4 Asp299Gly polymorphism	7.63 (2.21–26.41)	0.002
Age (≥50 years)	3.04 (1.09–6.85)	0.03
Systolic blood pressure (≥150 mm Hg)	1.62 (0.72–3.67)	NS

Legend: The model also included menopause status, diabetes mellitus, diastolic blood pressure (≥90 mm Hg) and use of calcium channel blockers, beta-blockers, angiotensin-converting enzyme inhibitors/angiotensin receptor blockers and diuretics as independent variables, which were not significantly associated with left ventricular hypertrophy. CI — confidence interval.

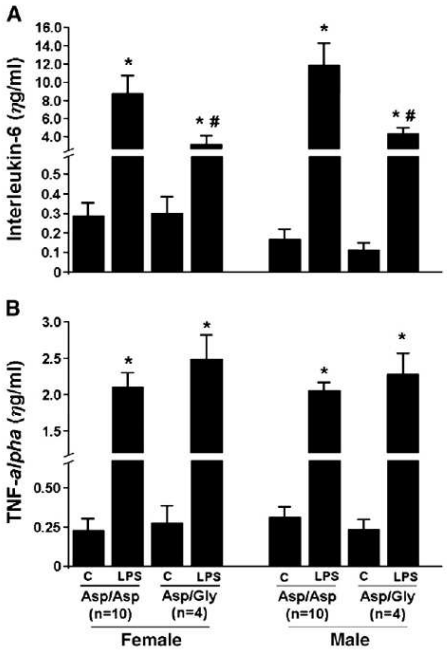

Fig. 1. Production of pro-inflammatory cytokines by monocytes of hypertensive patients with distinct TLR4 (Asp299Gly) genotypes. Results represent the mean ± standard error. C, unstimulated cells; LPS, lipopolysaccharide; *$p<0.05$ in comparison to sex-matched unstimulated cells; #$p<0.05$ in comparison to sex-matched LPS-stimulated cells with the Asp/Asp genotypes.

menopause status in the relationship between LV mass and the 299Gly allele seem to weaken this assumption. Therefore, further studies are necessary to determine the precise mechanisms by which TLR4 Asp299Gly might influence LV phenotype in women.

In the present report, heterozygous monocytes from hypertensive subjects of both genders exhibited a lower production of interleukin-6 after been stimulated with lipopolysaccharide. These findings are in accordance with data obtained in healthy subjects [28] as well as in individuals with osteomyelitis [9] and rheumatoid arthritis [19], demonstrating a decreased interleukin-6 response to lipopolysaccharide in leucocytes heterozygous for the 299Gly allele. Furthermore, they corroborate with results from other studies showing that epithelial cells from heterozygous subjects were functionally hyporesponsive [6,8]. Conversely, our results also showed no influence of the polymorphism in the induction of tumor necrosis factor-α in cultured monocytes. Accordingly, Montes et al. found no impact of the polymorphism on lipopolysaccharide-induced expression of tumor necrosis factor-α in heterozygous subjects with osteomyelitis [9], while other authors have shown that the influence of the 299Gly allele might be restricted to specific inflammatory markers [28], supporting the notion that this polymorphism may be associated with different cytokine responses to lipopolysaccharide stimulation.

The results shown herein revealed a significant lower prevalence of diabetes mellitus in female 299Gly carriers. This finding seems to corroborate with animal model studies showing diminished insulin resistance in association with defective TLR4 function [29]. However, contradictory data have been published regarding the impact of TLR4 polymorphisms on type 2 diabetes mellitus. In this regard, Illig et al. observed no association between Asp299Gly variant and diabetes

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mellitus [30], while Kolz et al. reported an association between other variations within the TLR4 gene and incident type 2 diabetes mellitus exclusively in men [31]. The reasons for such discrepancies are not clear, but it is possible that differences in protocol designs and patient characteristics could account for the contrasting results among the studies. In addition, it should be acknowledged that we did not routinely perform oral glucose tolerance test, a fact that might contribute to underestimate the prevalence of diabetes mellitus in our sample. On the other hand, the significant relationship between diabetes mellitus and TLR4 Asp299Gly polymorphism described herein seemed to explain the lower urinary albumin/creatinine ratio detected in female 299Gly carriers, indicating that this variant exerted no direct impact on hypertensive renal damage.

Some limitations to our study need to be addressed. First, the patients were pre-selected as data were collected in a tertiary referral clinic. Second, the majority of hypertensive subjects were on medications. Some findings might be, therefore, attributable to differential effect of various therapy regimens. However, we diminished this potential bias by considering in multivariate models the presence of antihypertensive medications. In addition, it was noteworthy that female 299Gly carriers presented a significant lower use of calcium channel blockers and angiotensin-converting enzyme inhibitors/angiotensin receptor blockers, thus strengthening the notion that use of antihypertensives played no major role in our results.

Our study group was not in Hardy–Weinberg equilibrium. Potential explanations for this finding may include small sample size and genotyping error. However, genotyping error is an unlikely reason because we evaluated the accuracy of genotyping in samples of all Gly carriers and of 90 randomly selected Asp/Asp subjects and there was no discordance in comparison to the former genotyping analysis. Another possible explanation could be the result of variations of genotype-associated disease risks. In our sample, deviation from Hardy–Weinberg equilibrium was solely detected in women. Noticeably, women carrying the Gly allele presented lower prevalence of left ventricular hypertrophy, which is an acknowledged risk factor for cardiovascular mortality [32]. Therefore, the lower prevalence of left ventricular hypertrophy in females carrying the Gly allele might potentially contribute to explain the higher prevalence of this allele in hypertensive women and the consequent deviation from Hardy–Weinberg equilibrium.

In conclusion, this study shows that genetic variation within the TLR4 gene is significantly associated with the extent of LV hypertrophy in female hypertensive patients. Regardless of the mechanisms, an association between TLR4 Asp299Gly polymorphism and LV mass in women with hypertension may be viewed in the context of epidemiologic data supporting sex differences in cardiac remodeling in pressure overload conditions. A more complete understanding of the biochemical processes by which TLR4 alters myocardial growth may aid in the discovery of novel approaches for the identification of women at high risk of developing LV hypertrophy.

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ORIGINAL CONTRIBUTIONS

Toll-Like Receptor 6 Ser249Pro Polymorphism Is Associated With Lower Left Ventricular Wall Thickness and Inflammatory Response in Hypertensive Women

Maria L. Sales[1,2], Roberto Schreiber[1], Maria C.S. Ferreira-Sae[1], Maruska N. Fernandes[1], Cristiane S.C. Piveta[1], José A.A. Cipolli[1], Cátia C. Cardoso[1], José R. Matos-Souza[1], Bruno Geloneze[1], Kleber G. Franchini[1] and Wilson Nadruz Jr[1]

BACKGROUND
Experimental data demonstrated that inactivation of toll-like receptor (TLR) pathway components attenuated left ventricular (LV) remodeling induced by pressure overload. This study investigated the impact of TLR6 Ser249Pro polymorphism on LV structure in hypertensive subjects.

METHODS
A sample of 443 patients (266 women and 177 men) was evaluated by clinical history, physical examination, analysis of inflammatory and metabolic parameters, echocardiography, and genotyping of the TLR6 variant. Moreover, the relationship between genotypes and *in vitro* responsiveness of peripheral blood monocytic cells to TLR agonists was also assessed.

RESULTS
Homozygous women for the TLR6 249Ser allele had lower LV posterior wall thickness (9.4 ± 0.4 vs. 10.5 ± 0.1 mm; $P = 0.02$), interventricular septum thickness (9.7 ± 0.3 vs. 10.7 ± 0.1 mm; $P = 0.03$), and LV relative wall thickness (0.39 ± 0.02 vs. 0.44 ± 0.01; $P = 0.02$) than women with other genotypes. These results were confirmed by stepwise regression analyses adjusted by potential confounders. Conversely, homozygous men for the 249Ser variant showed no differences in LV structure in comparison to males carrying the 249Pro allele. In addition, monocytes from hypertensive women homozygous for the 249Ser allele showed a lower release of tumor necrosis factor-α and interleukin-6 in response to zymosan (TLR6 agonist), but not to lipopolysaccharide (TLR4 agonist).

CONCLUSION
These data suggest that hypertensive women homozygous for the TLR6 249Ser polymorphism might exhibit lower LV wall thickness and reduced TLR6-mediated inflammatory response than females carrying the major allele.

Keywords: *blood pressure; gender; hypertension; left ventricle; polymorphism; toll-like receptor*

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Toll-like receptors (TLRs) are an important link between innate immunity and a variety of clinical disorders, including cardiovascular diseases.[1,2] Experimental studies have shown that TLRs might be involved in the development of left ventricular (LV) remodeling induced by pressure overload. For instance, TLR4 knockout mice developed less-severe LV remodeling following aortic banding than matched wild-type animals,[3] whereas blockade of MyD88, an intermediate factor of TLR pathway, also attenuated load-induced cardiac growth.[4] In addition, recent evidence revealed that TLRs mediate LV remodeling and dysfunction in ischemic hearts,[5,6] strengthening the concept that these receptors might be key regulators of cardiac structure following myocardial injury.

There are currently 11 known human TLRs, each of them contributing to specific recognition of particular pathogen-associated molecular patterns.[1] TLR6 forms heterodimers with TLR2 for recognition of microbial components and activates intracellular signaling pathways related to inflammatory response and cell growth.[7] Previous studies identified a coding polymorphism in the *TLR6* gene that leads to an exchange from serine to proline at position 249 in the extracellular domain of the protein.[8] This variant was associated with a reduced risk of asthma[8,9] and an increased risk of invasive aspergillosis,[10] but was not related to myocardial infarction.[8] Nevertheless, no

The first two authors contributed equally to this work.

[1]Department of Internal Medicine, School of Medicine, University of Campinas, Campinas, Brazil; [2]Department of Medical Sciences, Federal University of Ouro Preto, Ouro Preto, Brazil. Correspondence: Wilson Nadruz Jr (wilnj@fcm.unicamp.br)

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studies have determined the impact of TLR6 polymorphisms on end-organ damage in patients with systemic hypertension.

Thus, the aim of the present report was to investigate whether the TLR6 Ser249Pro polymorphism was associated with variation in LV structure in hypertensive subjects.

METHODS

Study population. The study was carried out in 443 unrelated hypertensive subjects, comprising 266 women and 177 men, followed up at the Hypertension Unit of a university hospital. Hypertension was defined as systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg or current antihypertensive medication use. Diabetes mellitus was diagnosed if fasting blood glucose was ≥126 mg/dl on two separate tests or when participants were taking hypoglycemic medications.[11] Women with reported amenorrhea for >12 months, except for pregnancy, were identified as postmenopausal. Coronary heart disease was diagnosed by history of myocardial infarction, acute coronary syndrome, or coronary revascularization, or by evidence of cardiac ischemia documented by functional testing. Main exclusion criteria were age under 18 years, significant cardiac valve disease, hypertrophic cardiomyopathy, and neoplastic disease. The research was carried out in accordance with the Declaration of Helsinki of the World Medical Association. The study was approved by the Ethics Committee of our institution, and written consent was obtained from all participants.

Blood pressure was measured using a validated digital oscillometric device (HEM-705CP; Omron Healthcare, Kyoto, Japan) with appropriate cuff sizes. Two readings were averaged and, if they differed by >5 mm Hg, one additional measurement was performed, and the average of the three measurements was taken.

Body mass index was calculated as body weight divided by height squared (kg/m^2), whereas waist circumference was measured at the midpoint between the lowest rib and iliac crest. Fasting blood total cholesterol, low-density lipoprotein-cholesterol, high-density lipoprotein-cholesterol, triglycerides, uric acid, glucose, insulin, and C-reactive protein levels were measured using standard laboratory techniques. The homeostasis model assessment index was calculated as follows: glucose (mg/dl) × insulin (μU/ml)/405 (ref. 12). In addition, albumin/creatinine ratio in morning urine samples and creatinine clearance were also measured.

Echocardiography. Echocardiography studies were performed on each subject at rest in the left lateral decubitus position using a Vivid 3 Pro (General Electric, Milwaukee, WI) apparatus equipped with a 2.5 MHz transducer as previously described.[13] LV end-diastolic and end-systolic diameters, interventricular septum thickness, posterior wall thickness, LV mass, and left atrial diameter were measured in accordance with the American Society of Echocardiography guidelines.[14] Relative wall thickness was computed as twice the posterior wall thickness divided by LV end-diastolic diameter. LV mass/height$^{2.7}$ was used to LV mass index determination.[15] All the recordings were made by the same physician, who was unaware of other data relating to the subjects. The reproducibility of both acquiring and measuring LV mass was determined in recordings obtained from 10 subjects. Intraobserver and interobserver LV mass variabilities were <8 and <11%, respectively.

Genotyping. Genomic DNA was extracted from peripheral blood leukocytes. The TLR6 Ser249Pro polymorphism was analyzed by polymerase chain reaction and digestion with Ava-II restriction enzyme.[9]

Cell culture and in vitro assays. In vitro stimulation of monocytic cells was performed as previously described,[16] with minor modifications. Briefly, peripheral blood monocytic cells from 30 hypertensive patients of different TLR6 Ser249Pro genotypes (10 Pro/Pro, 10 Pro/Ser, and 10 Ser/Ser subjects) were isolated by density gradient centrifugation over Histopaque 1077 and washed twice with phosphate-buffered saline. Cells were resuspended at 2×10^6 cells/ml and incubated in 1 ml of RPMI containing 10% fetal calf serum in 24-well tissue culture plates. Monocytes were isolated by adherence for 2 h in a humidified 5% CO_2 incubator at 37 °C. Cells were then stimulated with 10 μg/ml zymosan (a TLR6 agonist) or 10 μg/ml lipopolysaccharide (a TLR4 agonist) for 24 h, and interleukin-6 and tumor necrosis factor-α in the supernatants were determined by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). The response of cells to these stimuli was quantified as the fold-increase in the release of cytokines by stimulated monocytes compared to nonstimulated cells. In addition, immunoblotting of unstimulated cell extracts was performed as previously described[17] in order to evaluate TLR6 protein expression. Cells were lysed in assay lysis buffer (1% Triton, 10 mmol/l Tris-HCl, pH 7.4, 10 μg/ml aprotinin, 1 mmol/l PMSF, 0.25 mmol/l sodium orthovanadate). Insoluble material was removed by centrifugation for 20 min at 11,000 *g*, and samples were boiled with loading buffer and resolved by SDS-PAGE. Immunoblotting was performed using a primary antibody against TLR6 (sc-30001; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C, followed by exposure to [125I]protein A (Amersham Biosciences UK, Little Chalfont, UK). Bands corresponding to protein were quantified by optical densitometry.

Statistical analysis. Descriptive statistical results are given as means ± s.e.m. χ^2 test was used to compare categorical variables and to test for Hardy–Weinberg disequilibrium. Unpaired *t*-test was used to compare continuous variables. Stepwise regression analyses evaluated the independent predictors of echocardiographic parameters. Differences regarding *in vitro* production of interleukin-6 and tumor necrosis factor-α were evaluated by one-way analysis of variance followed by the Tukey test for pairwise comparisons. A P value of <0.05 was considered significant.

RESULTS

The frequency of TLR6 Ser249Pro genotypes is presented in **Table 1**. χ^2 analyses revealed that the polymorphism

distribution was in Hardy–Weinberg equilibrium in the studied population.

The clinical and laboratory characteristics of hypertensive subjects are shown in **Table 2**. In order to enhance statistical power, Ser/Pro subjects were added to Pro/Pro ones. No differences regarding the clinical and laboratory features were detected in hypertensive patients according to the genotype groups.

Echocardiography data are demonstrated in **Table 3**. Homozygous women for the 249Ser allele presented lower LV posterior wall thickness, interventricular septum thickness, and relative wall thickness than females exhibiting the major allele. Conversely, no significant association between the TLR6 variant and echocardiographic features was detected in hypertensive men.

Table 1 | Genotype frequencies of TLR6 Ser249Pro polymorphism

Genotype	Men (*n* = 177)	Women (*n* = 266)	Total (*n* = 443)
Pro/Pro, *n* (%)	103 (58)	152 (57)	255 (58)
Ser/Pro, *n* (%)	64 (36)	102 (38)	166 (37)
Ser/Ser, *n* (%)	10 (6)	12 (5)	22 (5)
Pro allele	0.763	0.764	0.763
Ser allele	0.237	0.236	0.237

Considering that the relationship between the TLR6 polymorphism and cardiac structure in hypertensive women could be influenced by potential confounders, stepwise regression analyses were performed (**Table 4**). The TLR6 249Ser/Ser genotype was independently related to posterior LV wall thickness and relative wall thickness in models that included age, body mass index, systolic blood pressure, diastolic blood pressure, menopause status, and antihypertensive medications as independent variables.

Given that the TLR6 249Ser/Ser genotype was associated with changes in LV mass solely in hypertensive women, we then evaluated whether there were gender-related differences in the inflammatory response between isolated monocytes from 249Pro carriers (Pro/Pro + Ser/Pro) and 249Ser/Ser hypertensive patients. In order to address this issue, peripheral blood monocytes were challenged *in vitro* with a TLR6 agonist (zymosan) or a TLR4 agonist (lipopolysaccharide), and the production of tumor necrosis factor-α and interleukin-6 was evaluated in the supernatant (**Figure 1a,b**). Monocytes homozygous for the 249Ser allele from hypertensive women, but not from hypertensive men, exhibited decreased zymosan-stimulated induction of tumor necrosis factor-α and interleukin-6, compared to sex-matched cells carrying the major allele. On the other hand, no difference in lipopolysaccharide-induced response was found between the

Table 2 | Clinical characteristics of hypertensive patients according to TLR6 Ser249Pro polymorphism

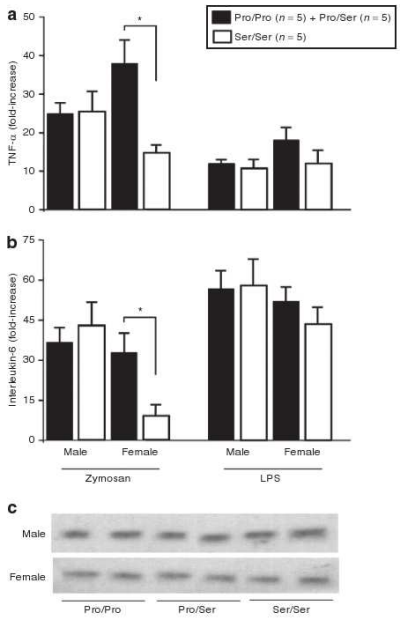
Characteristics	Men			Women		
Genotype	Pro/Pro + Ser/Pro	Ser/Ser	*P*	Pro/Pro + Ser/Pro	Ser/Ser	*P*
N	167	10		254	12	
Age, years	58.6 ± 1.0	54.1 ± 3.0	0.27	55.8 ± 1.0	60.1 ± 5.1	0.28
Body mass index, kg/m^2	30.1 ± 0.4	32.1 ± 1.7	0.24	31.9 ± 0.4	29.3 ± 1.8	0.14
SBP, mm Hg	148 ± 2	145 ± 5	0.70	150 ± 2	143 ± 8	0.32
DBP, mm Hg	85 ± 1	89 ± 4	0.48	87 ± 1	85 ± 2	0.49
Diabetes mellitus, *n* (%)	47 (28)	2 (20)	0.80	71 (28)	2 (17)	0.39
Smokers, *n* (%)	25 (15)	1 (10)	0.66	18 (7)	0 (0)	0.34
Coronary heart disease, *n* (%)	45 (27)	2 (20)	0.62	24 (9)	0 (0)	0.26
Postmenopause, *n* (%)	—	—		190 (75)	9 (75)	0.99
LDL-cholesterol, mg/dl	106 ± 3	120 ± 12	0.29	115 ± 2	113 ± 13	0.88
HDL-cholesterol, mg/dl	47 ± 1	49 ± 4	0.57	55 ± 1	52 ± 3	0.49
Triglycerides, mg/dl	168 ± 9	166 ± 16	0.97	148 ± 5	146 ± 14	0.89
HOMA	3.4 ± 0.3	4.2 ± 1.5	0.51	4.0 ± 0.3	3.2 ± 0.4	0.24
Uric acid, mg/dl	6.6 ± 0.1	6.2 ± 0.5	0.49	5.5 ± 0.1	6.2 ± 0.8	0.17
C-reactive protein, mg/dl	0.54 ± 0.09	0.43 ± 0.09	0.77	0.63 ± 0.05	0.54 ± 0.12	0.69
Log albumin/creatinine ratio, g/g	0.82 ± 0.05	0.84 ± 0.23	0.96	0.76 ± 0.04	0.79 ± 0.11	0.86
Creatinine clearance rate, ml/min	89 ± 3	101 ± 13	0.28	89 ± 2	95 ± 9	0.51
Diuretics, *n* (%)	131 (79)	8 (80)	0.93	206 (81)	9 (75)	0.60
CCB, *n* (%)	73 (44)	5 (50)	0.71	127 (50)	4 (33)	0.26
β-Blockers, *n* (%)	90 (54)	5 (50)	0.80	122 (48)	8 (66)	0.21
ACEI or ARB, *n* (%)	134 (81)	8 (80)	0.96	206 (81)	8 (66)	0.22

ACEI, angiotensin-converting enzyme inhibitors; ARB, angiotensin receptor blockers; CCB, calcium channel blockers; DBP, diastolic blood pressure; HDL, high-density lipoprotein; HOMA, homeostasis model assessment index; LDL, low-density lipoprotein; SBP, systolic blood pressure.

Table 3 | Echocardiographic features of hypertensive patients according to TLR6 Ser249Pro polymorphism

Variable	Men		*P*	Women		*P*
Genotype	Pro/Pro + Ser/Pro (*n* = 167)	Ser/Ser (*n* = 10)		Pro/Pro + Ser/Pro (*n* = 254)	Ser/Ser (*n* = 12)	
Interventricular septum, mm	11.1 ± 0.1	11.7 ± 0.5	0.29	10.7 ± 0.1	9.7 ± 0.4	0.03
Posterior wall thickness, mm	11.0 ± 0.1	11.2 ± 0.5	0.76	10.5 ± 0.1	9.4 ± 0.4	0.02
LV end-diastolic diameter, mm	53.5 ± 0.6	52.8 ± 1.4	0.75	48.5 ± 0.3	49.5 ± 1.8	0.52
Relative wall thickness	0.417 ± 0.006	0.425 ± 0.018	0.76	0.440 ± 0.005	0.390 ± 0.020	0.02
LV mass/height$^{2.7}$, g/m$^{2.7}$	76.0 ± 1.8	76.4 ± 6.0	0.96	74.7 ± 1.6	69.6 ± 5.6	0.48
Left atrium, mm	42.1 ± 0.5	41.9 ± 1.7	0.91	38.4 ± 0.3	36.6 ± 1.3	0.20

LV, left ventricular.



[Q1] **Figure 1 |** (**a,b**) Production of proinflammatory cytokines by monocytes of hypertensive patients with distinct TLR6 Ser249Pro genotypes. Results represent the mean ± s.e.m. (**c**) Representative blots of TLR6 expression in [Q2] unstimulated monocytes. LPS, lipopolysaccharide. *$P < 0.05$.

studied genotype groups of both genders. In addition, similar TLR6 protein expression was detected in unstimulated monocytes from all studied genotypes (**Figure 1c**).

DISCUSSION

Experimental evidence showed that TLR signaling pathway plays a major role in LV remodeling induced by pressure overload.[3,4] In the present report, we found that a TLR6 variant (Ser249Pro) was associated with lower LV wall thickness and relative wall thickness in hypertensive women. In addition, *in vitro* assays demonstrated that peripheral blood monocytes from women homozygous for the 249Ser allele presented a reduced release of tumor necrosis factor-α and interleukin-6 in response to a TLR6 agonist than sex-matched monocytes carrying the major allele. Taken together, these findings suggest that the coding TLR6 249Ser polymorphism might be associated with reduced TLR6-mediated inflammatory activity as well as lower LV wall thickness and relative wall thickness in hypertensive women. Given that increases in relative wall thickness are related to worse cardiovascular outcomes independently of LV mass,[18,19] it is possible that the present results might be of clinical relevance.

Table 4 | Stepwise regression analyses for selected independent variables and echocardiographic parameters in hypertensive women

Variable	R^2 change	*P*
Dependent: posterior wall thickness		
Body mass index	0.199	<0.0001
Systolic blood pressure	0.061	<0.0001
Age	0.013	0.03
TLR6 Ser/Ser = 0; TLR6 Ser/Pro + Pro/Pro = 1	0.011	0.04
Dependent: relative wall thickness		
Systolic blood pressure	0.054	0.0001
Body mass index	0.027	0.006
TLR6 Ser/Ser = 0; TLR6 Ser/Pro + Pro/Pro = 1	0.012	0.04

All models included age, body mass index, systolic blood pressure, diastolic blood pressure, TLR6 Ser249Pro genotypes, diabetes mellitus and use of diuretics, angiotensin-converting enzyme inhibitors/angiotensin receptor blockers, β-blockers, and calcium channel blockers as independent variables. Only variables with significant association were presented.

Human data demonstrated sex-related differences in LV structure and remodeling that are particularly manifested in pressure overload states.[20,21] These discrepancies have been partially explained by variations in sexual hormone profile,[21] body mass composition,[22,23] and metabolic factors.[23] Emerging evidence has suggested that inherited traits might also play a role in this regard. For instance, polymorphisms in the estrogen receptor β gene were associated with LV mass and wall thickness exclusively in women,[24] whereas variation at the

androgen receptor gene showed significant association with LV hypertrophy in males with hypertrophic cardiomyopathy.[25] The results shown herein indicated that TLR6 Ser249Pro polymorphism interacted with LV remodeling solely in hypertensive women. In addition, they are in agreement with recent experimental and clinical data derived from noncardiovascular diseases showing gender-related differences in TLR-related responses.[26–29]

To date, there have been no *in vitro* studies assessing the impact of TLR6 Ser249Pro polymorphism on inflammatory response. Here, we found that monocytes from hypertensive women carrying the Ser/Ser genotype exhibited diminished zymosan-induced cytokine release than sex-matched cells carrying the Pro allele. Conversely, no variation in the inflammatory response to zymosan was detected in hypertensive men according to TLR6 Ser249Pro genotypes. In addition, the polymorphism did not influence the response to lipopolysaccharide in both genders. Given that zymosan is an agonist of TLR6 pathway and lipopolysaccharide activates the TLR4 pathway,[1] these findings suggest that the TLR6 249Ser polymorphism might influence TLR6-related inflammatory response in women. The next step was to evaluate whether the variant was associated with changes in TLR6 protein expression in monocytes. In this context, immunoblotting assays revealed that protein content was not affected by the Ser249Pro variant, indicating that diminished inflammatory response observed in zymosan-stimulated Ser/Ser monocytes were not related to changes in TLR6 protein expression.

The reasons by which the Ser/Ser genotype associated with changes in LV structure and inflammatory response solely in hypertensive women are not apparent, even though potential explanations may include variation in sexual hormone profile and genetic background. In this regard, it is possible that chronic estrogen exposition interacted with TLR6 249Ser allele, thus modulating LV structure in hypertensive women. However, our results showing no differences in the prevalence of menopause between the genotype groups of hypertensive women seem to weaken this assumption. On the other hand, although the molecular pathways linking TLR6 to LV cardiac remodeling have not been previously explored, the fact that TLR6 activates the Myd88-NFκB[30] and PI3-kinase-AKT[31] pathways might provide potential connections between TLR6 activation and hypertension-induced cardiac remodeling.[1,4] Nevertheless, further studies are necessary to unveil the mechanisms by which TLR6 Ser249Pro interacts with gender and modulates inflammatory response and cardiac phenotype in hypertensive subjects.

Some limitations to our study need to be addressed. First, the population was preselected as data were collected in a tertiary referral clinic. Second, the majority of hypertensive patients were on medications. Some findings might be, therefore, attributable to differential effect of various therapy regimens. However, we diminished this potential bias by considering in multivariate models the presence of antihypertensive medications.

Overall, the results of the present study indicate that genetic variation within the *TLR6* gene is associated with variation in LV wall thickness in female hypertensive patients. Regardless of the mechanisms, an association between TLR6 Ser249Pro polymorphism and LV structure in women with hypertension may be viewed in the context of epidemiologic data supporting sex differences in cardiac remodeling in pressure overload conditions. Nevertheless, further studies in larger populations are needed to confirm the current evidence.

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IV. DISCUSSÃO

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Os receptores *Toll-like* são receptores transmembrana que ativam a resposta imune inata face a certos padrões moleculares de patógenos como também a fragmentos de tecido endógeno. Dados experimentais indicam que vias dos receptores Toll-like desempenham um papel importante na hipertrofia ventricular induzida por sobrecarga pressórica (19, 20).

Em nosso estudo, encontramos que a variante 299Gly de TLR4 foi associada com menor espessura de paredes de ventrículo esquerdo, menor índice de massa ventricular e menor prevalência de hipertrofia de ventrículo esquerdo em mulheres hipertensas, assim como a variante 249Ser de TLR6 também se associou com menor espessura de paredes ventriculares e menor espessura relativa de ventrículo esquerdo em hipertensas homozigotas. Estes resultados foram confirmados após análise de regressão logística, cujos fatores considerados como confundidores foram idade, índice de massa corpórea, *diabetes mellitus*, níveis de pressão arterial, uso de anti-hipertensivos e menopausa. De maneira geral, esses dados sugerem que pode haver uma interação entre gêneros, polimorfismos dos receptores Toll-like e fenótipo ventricular em indivíduos hipertensos.

Diferenças na geometria ventricular relacionadas ao gênero, como encontramos em nossos resultados, já foram relatadas na literatura. Estudo com participantes do *Framingham Heart Study* acompanhados de 1979 a 1993 mostrou que mulheres com hipertensão arterial sistêmica exibiram aumento da espessura das paredes e massa de ventrículo esquerdo sem alargamento da câmara (padrão de

hipertrofia concêntrica), enquanto os homens apresentaram dilatação do ventrículo esquerdo e aumento da massa do VE, sem aumento da espessura da parede (padrão de hipertrofia excêntrica) (55).

Possíveis explicações para essas diferenças foram levantadas, tais como a influência da variação da composição corporal, na qual após comparação de homens e mulheres obesos e hipertensos, observou-se que hipertensas obesas apresentavam hipertrofia ventricular mais pronunciada do que homens hipertensos com obesidade (56); a variação dos níveis de renina plasmática e de fator natriurético atrial, que sugerem a expansão do volume plasmático relacionado com o processo de envelhecimento nas mulheres, também tem sido apontada como provável explicação (56); são consideradas ainda como possibilidades as influências de hormônios sexuais (57) e de fatores metabólicos (58). Fatores genéticos como polimorfismos do gene receptor de estrógeno β, também foram associados com a massa de ventrículo esquerdo e com a espessura da parede ventricular em mulheres (59), enquanto variantes do gene do receptor de andrógeno mostraram associação significativa com hipertrofia ventricular em homens com cardiomiopatia hipertrófica (60).

Nosso estudo traz evidências de que os polimorfismos Asp299Gly e Ser249Pro foram associados com menor massa ventricular exclusivamente em mulheres, achados estes, que estão em acordo com recentes dados experimentais e clínicos que demonstraram que os receptores *Toll-like* podem exercer efeitos diferentes de acordo com o gênero em doenças não relacionadas ao sistema cardiovascular (61, 62). Em modelos animais, um estudo mostrou diferenças na produção de IL-6, um produto final da via do TLR 4, entre ratos machos e fêmeas (61). Em outro trabalho, foi comprovado que machos expostos aos LPS (um agonista de TLR4) produzem mais

IL-6 do que fêmeas após o mesmo estímulo, indicando uma explicação para a maior suscetibilidade de ratos machos à sepse bacteriana (63). Estudos com pacientes portadores de hepatite C crônica, mostrou distribuição diferente de variantes de TLR7 entre homens e mulheres com hepatite C crônica, e pior resposta ao tratamento com interferon, no grupo das mulheres com hepatite crônica portadoras da variante A>T (62). Mulheres portadoras do vírus HIV produzem mais interferon-α do que homens portadores deste vírus em resposta ao agonista de TLR7, o que pode explicar a progressão mais rápida das mulheres para AIDS, mesmo com carga viral semelhante à dos homens (64). Homens e mulheres portadores dos polimorfismos de TLR4 expostos a contaminantes provenientes da criação de suínos apresentaram diferenças na produção de interleucina-6 (IL-6) e de fator de necrose tumoral-alfa (TNF-α) entre (65).

Os mecanismos pelos quais os TLRs podem modular o desenvolvimento de hipertrofia cardíaca induzido por sobrecarga hemodinâmica ainda não estão completamente estabelecidos. Contudo, a ativação de algumas vias de sinalização celular poderia potencialmente explicar este processo. As proteínas TLR4 e TLR6, quando ativadas, iniciam uma via de sinalização intracelular dependente da proteína adaptadora MyD88, via que culmina com a produção de fator de necrose tumoral-alfa (TNF-α) e interleucina-6 (IL-6) (23). Estas citocinas, por sua vez, parecem exercer um papel central na fisiopatologia da hipertrofia ventricular esquerda, como remodelamento da matriz extracelular, proliferação de fibroblastos e hipertrofia de cardiomiócitos (66, 67).

As razões pelas quais os polimorfismos de TLR 4 e 6 estão associados com mudanças na estrutura ventricular esquerda exclusivamente em mulheres hipertensas

não são aparentes, ainda que as explicações possíveis possam incluir variação no perfil dos hormônios sexuais e alterações genéticas. Neste sentido, é possível que haja uma interação entre a exposição crônica de estrógeno e os alelos TLR6 249Ser e TLR4 299Gly, modulando a estrutura ventricular em mulheres hipertensas, apesar de nossos resultados não mostrarem diferenças na prevalência da menopausa entre os grupos de genótipos de mulheres hipertensas.

Contudo, não podemos descartar um efeito sinérgico envolvendo estrógenos e TLRs sobre o remodelamento ventricular. Neste contexto, estudo experimental realizado em ratos, no qual foi realizada a administração de estrógeno para ratos machos, levou ao declínio na produção de IL-6. Por outro lado, no mesmo estudo, quando foram realizadas ooforectomias em ratas fêmeas, com consequente diminuição dos níveis plasmáticos de estrógeno, houve aumento da produção desta interleucina, dados que sugerem que o estrógeno pode levar a inibição da produção de IL-6 (61).

No presente trabalho, estudos *in vitro* com monócitos retirados do sangue periférico mostraram que pacientes hipertensos portadores da variante TLR4 299Gly e mulheres hipertensas portadoras do TLR6 249Ser apresentaram menor produção de IL-6 quando estimulados com seus respectivos agonistas (LPS para o TLR4 e zimosan para o TLR6). Em concordância com nossas observações, dados de outros grupos também demonstraram que o polimorfismo TLR4 Asp299Gly se acompanha de menor produção *in vitro* de IL-6 em indivíduos saudáveis (68), portadores de osteomielite (69), e indivíduos com artrite reumatóide (70). Por outro lado, nossos resultados são os primeiros da literatura a demonstrar que o polimorfismo TLR6 249Ser pode ser funcional *in vitro*.

Esses dados em conjunto podem sugerir que, em mulheres portadoras destes polimorfismos, há uma dupla inibição da produção de IL-6, seja a inibição causada pela exposição ao estrógeno, seja pela inibição da via dos receptores *Toll-like*.

Além da redução da produção de IL-6 nos portadores da variante 299Gly e em homozigotas para o alelo 249Ser, os dados da presente pesquisa também mostraram menor produção de TNF-α em monócitos de mulheres homozigotas para 249Ser estimulados com zymosan. Estudos sugerem que a IL-6 está envolvida com a hipertrofia cardíaca, atuando tanto na hipertrofia de cardiomiócitos quanto na proliferação de fibroblastos cardíacos (71, 72). O TNF-α também atua no desenvolvimento da hipertrofia ventricular (73).

A produção diminuída da IL-6 e de TNF-α nos portadores dos polimorfismos estudados neste trabalho pode ter um papel importante nas alterações ventriculares observadas, porém novos estudos ainda se mostram necessários para completar a complexa fisiopatologia envolvida na relação dos TLRs, gêneros e alterações por sobrecarga pressórica na estrutura do ventrículo esquerdo.

Os resultados aqui apresentados também revelaram uma prevalência significativamente menor de *diabetes mellitus* em mulheres portadoras do alelo 299Gly.

Este achado parece confirmar dados de estudo em modelo animal que mostra sensibilidade aumentada à insulina em ratos com mutação que leva a perda de função do TLR 4, além de menor obesidade induzida por dieta nestes animais (74). Contudo, dados contraditórios têm sido publicados sobre o impacto dos polimorfismos de TLR4 e *diabetes mellitus* tipo 2. A este respeito, Illig *et al.* não observaram nenhuma associação entre o polimorfismo Asp299Gly e *diabetes mellitus* (75), enquanto Kolz *et*

al. relataram uma associação entre variantes do gene TLR4 e incidência de *diabetes mellitus* tipo 2 exclusivamente em homens (76). As razões para tais discrepâncias não são claras, mas é possível conjecturar que as diferenças nos desenhos dos estudos e das características dos pacientes podem ser responsáveis pelos resultados contrastantes encontrados. Além disso, deve-se reconhecer que não foi realizado rotineiramente o teste oral de tolerância à glicose, fato que pode contribuir para subestimar a prevalência de *diabetes mellitus* em nossa amostra.

A teoria de que a inflamação em tecidos metabólicos pode causar o desenvolvimento de resistência à insulina originou-se com a descoberta de que o tecido adiposo era capaz de produzir TNF-alfa, citocina inflamatória que prejudica a via de sinalização da insulina. Posteriormente, desvendou-se que outras citocinas inflamatórias subjacentes ao TNF-alfa provocariam resistência à insulina induzida por obesidade (77).

Recentes estudos evidenciam que ácidos graxos provenientes da dieta, principalmente os de origem animal, são capazes de ativar a proteína TLR-4, que funciona como mediador da via inflamatória com consequências negativas para ações da insulina em tecidos metabólicos.

Esse receptor desempenha uma conexão importante entre o sistema imune inato e o sistema metabólico (78). A ingestão elevada de gordura na dieta pode ativá-lo mesmo na ausência de patógenos, desencadeando uma resposta inflamatória capaz de interferir nos sinais mediados pelos hormônios controladores da fome e do gasto energético, resultando em obesidade e resistência à insulina (79).

Apesar dos dados experimentais sinalizarem a relação dos receptores *Toll-like* com a obesidade, os resultados para os polimorfismos aqui estudados (Asp299Gly de

TLR 4 e Ser249Pro de TLR 6) não demonstraram diferença significativa quanto à prevalência de obesidade entre os grupos.

Algumas limitações do nosso estudo devem ser abordadas. Primeiro, como os dados foram coletados em um centro de referência terciário, os pacientes foram pré-selecionados. Em segundo lugar, a maioria dos indivíduos hipertensos estava em uso de medicações. Alguns resultados poderiam ser, portanto, devido ao efeito dos diferentes regimes de terapia adotados. No entanto, tentamos diminuir este potencial viés, considerando em modelos de análise multivariada a presença de medicamentos anti-hipertensivos.

A distribuição do polimorfismo Asp299Gly do receptor Toll-like 4 não está em equilíbrio de Hardy-Weinberg. Consideramos que poderia tratar-se de erro de genotipagem. Porém ao refazermos as reações dos indivíduos portadores do alelo Gly e de 90 amostras aleatoriamente selecionadas, não encontramos divergências nesses resultados. Outra possível explicação poderia ser o fato das mulheres portadoras do alelo Gly apresentarem menor hipertrofia de ventrículo esquerdo, com consequente menor mortalidade, o que levaria ao aumento da prevalência desse alelo e desvio do equilíbrio de Hardy-Weinberg. Por fim, não podemos descartar que a ausência de equilíbrio de Hardy-Weinberg possa se dever ao tamanho relativamente pequeno de nossa amostra.

V. CONCLUSÃO

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As principais conclusões desse trabalho são:

Mulheres que apresentam o alelo 299Gly de TLR 4 apresentam menor parede posterior de ventrículo esquerdo, menor septo interventricular, menor índice de massa de ventrículo esquerdo e reduzida prevalência de hipertrofia de ventrículo esquerdo.

Hipertensas homozigotas para o alelo 249Ser de TLR 6 apresentam menor parede posterior de ventrículo esquerdo, menor septo interventricular, menor espessura relativa de ventrículo esquerdo.

Os dados *in vitro* permitem inferir que monócitos de mulheres com o alelo 299Gly produziram menos IL-6 quando em resposta ao estímulo por LPS.

Monócitos de mulheres homozigotas para o alelo Ser249 mostraram menor produção de IL-6 e TNF-α em resposta ao zymosan.

Esses dados sugerem que pode haver uma interação entre gêneros, polimorfismos dos receptores Toll-like e fenótipo ventricular em indivíduos hipertensos.

VI. REFERÊNCIAS BIBLIOGRÁFICAS

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