

Universidade Federal do Rio Grande do Sul

Programa de Pós-graduação em Ciências Médicas: Endocrinologia

**ASSOCIAÇÃO ENTRE OS POLIMORFISMOS ASP299GLY E THR399ILE NO
GENE DO RECEPTOR DO TIPO TOLL-4 (*TLR4*) E O DIABETES MELLITUS
TIPO 2: ESTUDO CASO-CONTROLE E META-ANÁLISE**

Dissertação de Mestrado

Natália Emerim Lemos

Porto Alegre, novembro 2014

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“Temos o destino que merecemos. O
nosso destino está de acordo com os
nossos méritos.”

Albert Einstein

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- **Artigo original:** Associação entre os polimorfismos Asp299Gly e Thr399Ile no gene do receptor do tipo Toll-4 (*TLR4*) e o Diabetes Mellitus tipo 2: Estudo Caso-Controle e Meta-análise (aceito para publicação pela revista *Journal of Diabetes & Metabolism* – Fator de impacto: 3,38).

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1. Introdução

DAMPs *Damage-associated molecular patterns*

DM Diabetes mellitus

DM2 Diabetes mellitus tipo 2

INF Interferon

IFN-1 Interferon 1

IFN- β Interferon β

IL-6 Interleucina 6

IL-1 β Interleucina 1 β

IP-10 *IFN-inducible protein 10*

iNOS *Inducible nitric oxide*

IRF-3 Fator regulador de interferon

LPS Lipopolissacarídeos

LRR *Leucine-rich repeat*

MAPK *Mitogen-activated protein kinase*

MyD88 Fator de diferenciação mielóide 88

NF-κB	Fator nuclear kappa B
NLRs	<i>NOD-like receptors</i>
NOD	<i>Nucleotide-oligomerization domain</i>
PAMPs	<i>Pathogen-associated molecular patterns</i>
PRRs	<i>Pattern-recognition receptors</i>
RC	Razão de chance
RIG-I	<i>Retinoic acid-inducible I</i>
RLHs	<i>RIG-I-like helicases</i>
TIR	Receptor Toll/IL-1
TLR1-10	<i>Toll-like receptor 1-10</i>
TLRs	<i>Toll-like receptors</i>
TNF	Fator de necrose tumoral
TRIF	Adaptador que contém domínio TIR

2. Artigo Original

AH	Arterial hypertension
BMI	Body mass index
BP	Blood pressure
CI	Confidence interval
LD	Linkage disequilibrium
DCCT	Diabetes control and complications trial
FEM	Fixed effect model
HbA1c	Glycated hemoglobin
HWE	Hardy-Weinberg equilibrium
IR	Insulin resistance
LPS	Lipopolysaccharide
MeSH	Medical subject headings
NF- κ B	Factor nuclear kappa B
OR	Odds ratio
PAMPs	<i>Pathogen-associated molecular patterns</i>
PRRs	<i>Pattern-recognition receptors</i>

REM	Random effect model
SD	Standard deviation
T2DM	Type 2 diabetes mellitus
TLR4	Toll-like receptor 4
TLRs	Toll-like receptors

RESUMO

Introdução: O Diabetes Mellitus tipo 2 (DM2) é uma doença crônica complexa causada pela combinação de resistência à insulina no músculo esquelético, fígado e tecido adiposo, e desregulação na secreção de insulina pelas células-beta pancreáticas. Tanto o DM2 quanto a resistência à insulina são considerados como estado de inflamação crônica pré-clínica, resultantes de mudanças na resposta imune inata. Dessa forma, polimorfismos em genes que codificam proteínas do sistema imune inato, como os receptores do tipo Toll, podem influenciar a resposta imunológica bem como desenvolvimento do DM2. Este trabalho descreve um estudo de caso-controle e uma meta-análise realizada para determinar se os polimorfismos Asp299Gly (rs4986790) e Thr399Ile (rs4986791) no gene *TLR4* estão associados com DM2.

Métodos: No estudo de caso-controle foram incluídos 1.684 pacientes com DM2 e 584 indivíduos não diabéticos do Brasil. A pesquisa bibliográfica foi realizada a fim de identificar os estudos que investigaram a associação entre os polimorfismos referidos no gene *TLR4* e DM2. As razões de chances (RC) dos grupos foram calculadas para os modelos de herança de contraste de alelos e dominante.

Resultados: No estudo de caso-controle, as frequências genótípicas e alélicas dos polimorfismos Asp299Gly e Thr399Ile diferiram entre pacientes com DM2 e indivíduos não diabéticos ($p < 0,05$). Além disso, a presença de alelos raros desses polimorfismos foi significativamente associada com proteção para DM2, após o ajuste para etnia, sob o modelo dominante [Asp299Gly: RC = 0,68 (IC 95% 0,49-0,94); Thr399Ile: RC = 0,65 (IC 95%,46-0,90)]. Sete estudos foram selecionados para inclusão na meta-análise. Resultados da meta-análise mostram que o

polimorfismo Asp299Gly foi associado à proteção para o DM2 [RC = 0,68 (IC 95% 0,46-1,00), modelo de contraste alelo]. A estratificação por etnia revelou que ambos os polimorfismos foram associados à proteção para DM2 sob os modelos de contraste de alelos e dominante na população brasileira, mas não em europeus.

Conclusões: Em nosso estudo de caso-controle, demonstramos uma possível associação entre os polimorfismos Asp299Gly e Thr399Ile no gene *TLR4* e proteção para DM2. Da mesma forma, os resultados da meta-análise mostraram uma associação do polimorfismo Asp299Gly com proteção para o DM2 em todos os grupos étnicos. No entanto, após estratificação por etnia, encontramos associação entre os dois polimorfismos estudados e proteção para o DM2 em brasileiros, mas não em descendentes de europeus. Essa é a maior meta-análise que investigou os polimorfismos Asp299Gly e Thr399Ile no gene *TLR4* até o presente momento. Em outras etnias são necessários novos estudos com amostras maiores para confirmar essas associações, bem como elucidar os papéis possivelmente desempenhados por esses polimorfismos na patogênese do DM2.

ABSTRACT

Introduction: Type 2 Diabetes Mellitus (T2DM) is a chronic disease caused by a complex combination of insulin resistance in skeletal muscle, liver and adipose tissue, and deregulation of insulin secretion by pancreatic beta-cells. Both T2DM and insulin resistance are considered as a state of chronic pre-clinical inflammation resulting from changes in the innate immune response. Therefore, polymorphisms in genes encoding proteins of the innate immune system, such as Toll-like receptors, can influence the immune response and the development of T2DM. This work describes a case-control study and a meta-analysis conducted to determine whether the *TLR4* Asp299Gly (rs4986790) and Thr399Ile (rs4986791) polymorphisms are associated with T2DM.

Methods: In the case-control study were enrolled 1684 T2DM patients and 584 nondiabetic subjects from Brazil. A literature search was conducted in order to identify studies that investigated associations between the referred *TLR4* polymorphisms and T2DM. Pooled odds ratios (OR) were calculated for allele contrast and dominant inheritance models.

Results: In the case-control study, genotype and allele frequencies of the Asp299Gly and Thr399Ile polymorphisms differed between T2DM patients and nondiabetic subjects ($P < 0.05$). Moreover, the presence of the minor alleles of these polymorphisms were significantly associated with protection for T2DM, after adjusting for ethnicity, under a dominant model [Asp299Gly: OR=0.68 (95% CI 0.49-0.94); Thr399Ile: OR=0.65 (95% CI 0.46-0.90)]. Seven studies were eligible for inclusion in the meta-analysis. Meta-analysis results showed that the Asp299Gly polymorphism was associated with T2DM protection [OR=0.68 (95% CI 0.46-1.00), allele contrast model]. Stratification by ethnicity revealed that both polymorphisms were associated with

T2DM protection under allele contrast and dominant models in Brazilian population but not in Europeans.

Conclusions: In our case-control study, we were able to demonstrate a possible association between the *TLR4* Asp299Gly and Thr399Ile polymorphisms and protection for T2DM. In agreement, the meta-analysis results showed an association of the Asp299Gly polymorphism with T2DM protection in the whole group, and associations of the Asp299Gly and Thr399Ile polymorphisms with T2DM protection in the Brazilian group but not in European descendent. This is the largest *TLR4* meta-analysis performed so far. In other ethnicities further studies with large sample size are necessary to confirm these associations as well as to elucidate the roles possibly played by these polymorphisms in the pathogenesis of T2DM.

INTRODUÇÃO

De acordo com a Federação Internacional de Diabetes (1) 285 milhões de indivíduos em todo o mundo apresentam algum tipo de diabetes mellitus (DM). Estatísticas mostram que o número de indivíduos afetados continua a aumentar e que, se não forem tomadas providências para modificar a trajetória da epidemia, a prevalência poderá chegar a 440 milhões de indivíduos com DM em 2030. O Brasil encontra-se em quinto lugar neste ranking com 7,6 milhões de casos de DM, sendo que cerca de 90% do total de casos apresenta DM tipo 2 (DM2).

O DM2 é uma doença crônica complexa causada pela combinação de resistência à insulina no músculo esquelético, fígado e tecido adiposo, e desregulação na secreção de insulina pelas células-beta pancreáticas (2, 3). De acordo com a hipótese da “metainflamação” (inflamação desencadeada pelo metabolismo), tanto o DM2 quanto a resistência à insulina são considerados como estados de inflamação crônica pré-clínica, resultantes de mudanças na resposta imune inata (4-8). Dessa forma, polimorfismos em genes que codificam proteínas do sistema imune inato, como os receptores do tipo *Toll-like receptors* (TLRs), podem influenciar a resposta imunológica bem como o desenvolvimento do DM2 (9).

A imunidade inata representa a primeira linha de defesa de humanos e outros organismos pluricelulares contra microrganismos patogênicos (10). O reconhecimento dos patógenos pelo sistema imune inato depende dos *Pattern recognition receptors* (PRRs). Estes receptores possuem a habilidade de identificar estruturas altamente conservadas na maioria dos patógenos, os *Pathogens-Associated Molecular Pattern* (PAMPs) (8, 11). Estudos recentes identificaram três grupos principais de PRRs: *Toll-like receptors* (TLRs), *retinoic acid-inducible gene 1* (RIG-I)-like helicases (RLHs) e

nucleotide-oligomerization domain (NOD)-like receptors (NLRs) (12). Entre os PRRs, os mais extensivamente estudados são os TLRs, que são glicoproteínas transmembranas originalmente identificadas em *Drosophila melanogaster* (8, 13). Além de responder aos PAMPs, TLRs também podem responder às moléculas que são liberadas a partir de tecidos ou células danificados, denominados *Damage-Associated Molecular Patterns (DAMPs)* (14). A ligação do TLR ao seu ligante desempenha um papel chave na imunidade inata e imunidade adquirida contra subsequente infecção microbiana ou alguma lesão tecidual ou celular (15).

Durante uma infecção (viral ou bacteriana), PAMPs específicos são reconhecidos (RNA de fita simples/dupla, DNA ou ainda componentes da parede celular bacteriana) pelos PRRs presentes nas células infectadas ou em macrófagos e células dendríticas, o que irá ativar vias sinalizadoras que regulam a produção de diversas citocinas, quimiocinas e interferon do tipo 1 (IFN-1). Esses interferons possuem uma potente ação antiviral, antiproliferativa e imunomoduladora e têm como objetivo proteger as células não infectadas e induzir apoptose naquelas já contaminadas (11, 12). Além disso, a ativação da imunidade inata é um passo importante para o desenvolvimento do sistema imune adaptativo específico para o antígeno. A ativação consiste em linfócitos B, que produzem anticorpos específicos contra o agente patogênico invasor, e em linfócitos T, que secretam citocinas que induzem a eliminação de células infectadas, exercendo efeitos citotóxicos ou sinalizando para os linfócitos B (16, 11).

Estruturalmente, TLRs são PRRs do tipo I de sinalização transmembrana, o seu domínio extracelular inclui uma estrutura repetitiva rica em resíduos de leucina, chamada *leucine-rich repeat (LRR)*, a qual está envolvida no reconhecimento do

ligante. Um domínio transmembrana determina a sua localização celular e a porção intracelular contém uma estrutura semelhante à do receptor da IL-1, conhecido como domínio Toll/IL-1 (TIR), que é essencial para o desencadeamento de vias de sinalização intracelulares (14, 15, 17). Até agora, onze TLRs foram identificados em seres humanos e 13 em ratos, reconhecendo diferentes PAMPs e DAMPs (16, 18, 12). A **Tabela 1** mostra os 11 TLRs humanos com seus ligantes específicos. Os ligantes para o TLR8 e o TLR10 ainda são desconhecidos (19). Os TLRs são localizados tanto na superfície celular quanto em compartimentos intracelulares. Os TLRs presentes na superfície celular, como TLR1, TLR2, TLR4, TLR5, TLR6 e TLR10, são essenciais para o reconhecimento de componentes da parede celular bacteriana, flagelos bacterianos, partículas virais, fungos. TLR3, TLR7, TLR8 e TLR9 são localizados em endossomos e seus ligantes, principalmente ácidos nucleicos virais ou bacterianos, requerem internalização nos endossomos (15,16, 20, 21).

Tabela 1. PAMPs reconhecidos pelos TLRs.

TLRs	Localização	Microrganismos	PAMPs reconhecidos
TLR1	Membrana plasmática	Bactéria	Lipoproteína Triacil
TLR2	Membrana plasmática	Bactéria	Lipoproteína
		Fungo	<i>Zymosan</i> , betaglicano
		Parasita	tGPI-mucina (<i>Trypanosoma</i> sp)
TLR3	Endolisossoma	Vírus	dsRNA
TRL4	Membrana plasmática	Bactéria	LPS
		Vírus	Proteína estrutural
		Parasita	Glico inositol fosfolípidios (<i>Trypanosoma</i> sp)
		Fungo	Manano
TLR5	Membrana plasmática	Bactéria	Flagelina
TLR6	Membrana plasmática	Bactéria	Lipoproteína Diacil

		Vírus	Lipoproteína Diacil
		Fungo	<i>Zymosan</i> , betaglucano
TLR7	Endolisossoma	Vírus	ssRNA
		Bactéria	RNA
		Fungo	RNA
TLR8	Endolisossoma	Desconhecido	Desconhecido
TLR9	Endolisossoma	Vírus	CpG-DNA
		Bactéria	DNA
		Fungo	DNA
		Parasita	Hemozoína (<i>Plasmodium</i> sp)
TLR10	Endolisossoma	Desconhecido	Desconhecido
TLR11	Membrana plasmática	Protozoário	Molécula do tipo Profilina

TLRs são amplamente expressos em várias células imunitárias, incluindo as células dendríticas, macrófagos, células B, tipos específicos de células T e mesmo em células não-imunes, tais como células epiteliais, células endoteliais e fibroblastos (22, 23). A expressão de TLRs não é estática, mas sim é regulada rapidamente em resposta a patógenos, uma variedade de citocinas, e estresses ambientais (23). Curiosamente, a expressão de TLRs em células não imunes parece ter um papel importante na primeira linha de defesa contra a invasão microbiana nesses locais (19).

Após ligação ao ligante, a sinalização dos TLRs ocorre através de duas vias: via mediada pelo fator de diferenciação mielóide 88 (MyD88), e outra via mediada pelo domínio TIR contendo adaptador que leva a indução de IFN- β (TRIF). O primeiro caminho faz com que a ativação do fator nuclear kappa B (NF- κ B) e várias proteínas quinases ativadas pelo *mitogen-activated protein kinase* (MAPK), induzam genes que contribuem para reações inflamatórias. Já a segunda via provoca indução de IFN, cuja estimulação leva a um estado anti-inflamatório. TLR3 só ativa a via mediada por TRIF,

que conduz à ativação do fator regulador de interferon (IRF)-3, um importante fator de transcrição, que induz a expressão de IFN-1 e a super-regulação de maneira indireta de genes dependentes de IFN, como o *IFN-inducible protein 10* (IP-10) e o *inducible nitric oxide* (iNOS). Além disso, TLR3 também ativa NF-κB, que regula positivamente a produção de citocinas pró-inflamatórias, tais como IL-1β, IL-6 e TNF. Já o TLR4, ativa ambas as vias, ativando NF-κB e induzindo a produção de IFN-1. Todos os outros *TLRs* ativam exclusivamente a via dependente de MyD88 (20, 22).

Resumidamente, as principais funções de TLRs são induzir uma resposta inflamatória e estabelecer imunidade adaptativa. TLRs ativam macrófagos teciduais, estimulando a produção de citocinas pró-inflamatórias, incluindo TNF, IL-1β e IL-6, que coordenam as respostas inflamatórias locais e sistêmicas. Estas citocinas, em seguida ativam as células circundantes para produzir quimiocinas ou moléculas de adesão, recrutando, assim, várias células inflamatórias para o local da inflamação. Macrófagos ou neutrófilos recrutados são ativados e fagocitam os patógenos invasores através da internalização dos PRRs. Depois, essas células destroem os agentes patogênicos através da produção de óxido nítrico, espécies reativas de oxigênio ou defensinas. (14, 23).

Diversos membros da família dos TLRs têm surgido como sensores-chaves da imunidade inata para o reconhecimento de componentes bacterianos, tais como lipopolissacarídeos (LPS) presentes na membrana de bactérias gram-negativas. A ligação do patógeno aos TLRs desencadeia a produção de IFN-1, citocinas pró-inflamatórias e quimiocinas e induz a resposta imune necessária para eliminar o patógeno. Além disso, através da ativação dos TLRs, a maturação de células dendríticas é induzida, iniciando, assim, a resposta imune adaptativa (24).

Diversos estudos têm demonstrado a associação do *TLR4* com resistência à insulina e DM2 (25, 26, 41-45). Manolakis *et al.* (25), verificaram que os alelos mutados dos polimorfismos Asp299Gly e Thr399Ile estão associados à proteção para o DM2. Arora P *et al.* (26), demonstraram que polimorfismos nos genes responsáveis pela imunidade inata estão associados com fatores que podem estar relacionados ao DM2.

Receptor do tipo Toll- 4 (TLR4)

O TLR4 reconhece como ligante o LPS bacteriano (27), e é expresso em macrófagos, tecido adiposo, músculo esquelético, pâncreas e endotélio vascular (28, 29). Esse receptor também interage com ligantes endógenos como ácidos graxos livres, *heat shock proteins* 60 e 70, fibrinogênio e fibronectina, os quais estão elevados em pacientes com DM2 (28, 30-32). Além disso, a ativação do TLR4 parece ter um importante papel na patogênese da resistência à insulina e DM2, tanto em condições clínicas quanto em condições experimentais, como demonstrado na **Figura 1** (28, 33-37).

O TLR4 possui um papel importante na inflamação e na imunidade, uma vez que há expressão desse receptor na maioria dos tecidos humanos, incluindo tecidos sensíveis à insulina. Por ser ativado por LPS e ácidos graxos saturados (34), que são indutores de resistência à insulina, TLR4 pode ser um candidato em comum na interação entre sinais inflamatórios e metabólicos.

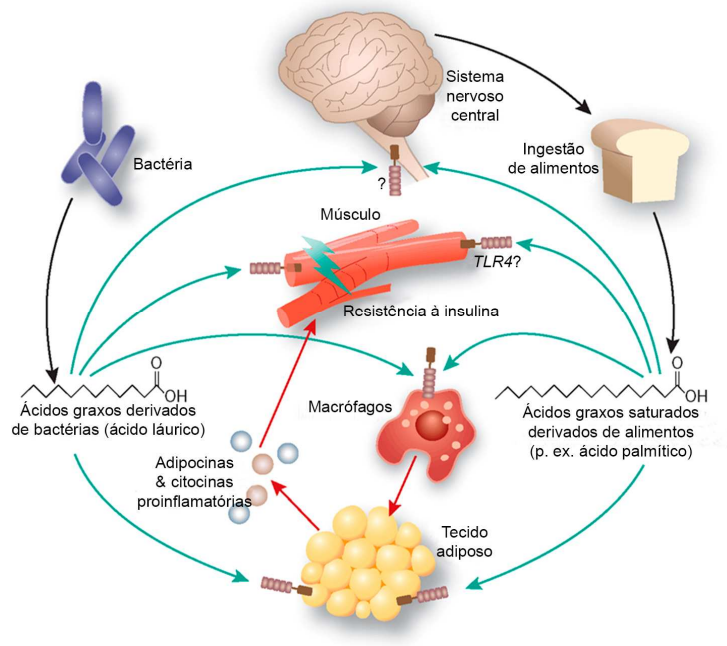


Figura 1. Receptores do tipo Toll, como TLR4 são importantes para mediar nossa resposta imune inata a patógenos bacterianos. Além disso, também desencadeia processos inflamatórios após exposição a certas gorduras dietéticas: TLR4 em macrófagos e tecido adiposo estimulam citocinas proinflamatórias, que por sua vez causam a resistência à insulina no músculo e provavelmente em outros tecidos. Estes achados sugerem que TLR4 pode ser um importante componente da via que liga a obesidade induzida por dieta com a inflamação, a resistência à insulina e diabetes. Além disso, TLR4 pode desempenhar um papel no sistema nervoso central, onde pode modular a ingestão de alimentos em resposta a agentes patogênicos bacterianos, bem como para certos lipídeos nutricionais. Figura adaptada (38).

O gene *TLR4* consiste em três exons e está localizado no cromossomo 9q32-32. Dois polimorfismos funcionais nesse gene (Asp299Gly e Thr399Ile) foram associados com um fenótipo hiporresponsivo a LPS (39). No polimorfismo Asp299Gly ocorre uma troca de base A para G, resultando em uma troca de aminoácidos, de aspartato para glicina. Já no polimorfismo Thr399Ile ocorre uma troca de base C para T, resultando em

uma troca de aminoácidos, de treonina para isoleucina. Os polimorfismos Asp299Gly e Thr399Ile estão em completo desequilíbrio de ligação em diferentes populações (40). Desde sua descrição, a associação desses polimorfismos com DM2 e resistência à insulina tem sido investigada; entretanto, os resultados têm sido contraditórios, como pode ser evidenciado na **Tabela 2** (25, 26, 41-45).

Tabela 2. Estudos que investigaram a associação dos polimorfismos no gene *TLR4* e DM2.

Estudo	Tipo associação descrita
Belforte FS <i>et al.</i> (41)	Polimorfismo Asp299Gly e a diminuição do risco para DM2 e Síndrome metabólica
Arora P <i>et al.</i> (26)	Polimorfismos em 5 genes da imunidade inata, incluindo o Asp299Gly, podem ser considerados fatores de risco metabólico com fenótipo de DM2
Buraczynska M <i>et al.</i> (42)	Os polimorfismos Asp299Gly e o Thr399Ile associados com aumento do risco de desenvolvimento precoce de retinopatia diabética em pacientes com o DM2
Manolakis AC <i>et al.</i> (25)	Polimorfismos Asp299Gly e Thr399Ile associados com proteção para o DM2
Maldonado-Bernal C <i>et al.</i> (43)	Polimorfismos Asp299Gly e Thr399Ile são mais frequentes em pacientes com DM2 do que em indivíduos saudáveis
Ilig T <i>et al.</i> (44)	Sem associação entre o polimorfismo Asp299Gly no gene <i>TLR4</i> com a síndrome metabólica e o DM2
Kolek MJ <i>et al.</i> (45)	Polimorfismo Asp299Gly associado com diminuição do risco para doença arterial coronariana e DM

Dessa forma, o objetivo do presente estudo foi verificar a associação entre os polimorfismos Asp299Gly e Thr399Ile no gene *TLR4* e o DM2, através de um estudo caso-controle de uma amostra do sul do Brasil, seguido de uma revisão sistemática e meta-análise de estudos da literatura.

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ARTIGO ORIGINAL

**ASSOCIAÇÃO ENTRE OS POLIMORFISMOS ASP299GLY E THR399ILE NO
GENE DO RECEPTOR DO TIPO TOLL-4 (*TLR4*) E DIABETES MELLITUS
TIPO 2: ESTUDO CASO-CONTROLE E META-ANÁLISE**

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**ASSOCIATION BETWEEN ASP299GLY AND THR399ILE
POLYMORPHISMS IN TOLL-LIKE RECEPTOR 4 GENE AND TYPE 2
DIABETES MELLITUS: CASE-CONTROL STUDY AND META-ANALYSIS.**

TLR4 polymorphisms and T2DM: case-control study and meta-analysis

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Highlights:

- *TLR4* polymorphisms are associated with protection for T2DM.
- Meta-analysis showed an association of Asp299Gly polymorphism with T2DM protection.
- This is the largest *TLR4* meta-analysis performed so far.

Abstract

Objective: This paper describes a case-control study and a meta-analysis conducted to determine whether the *TLR4* Asp299Gly (rs4986790) and Thr399Ile (rs4986791) polymorphisms are associated with type 2 diabetes mellitus (T2DM).

Methods: In the case-control study were enrolled 1684 T2DM patients and 584 nondiabetic subjects from Brazil. A literature search was conducted in order to identify studies that investigated associations between the referred *TLR4* polymorphisms and T2DM. Pooled odds ratios (OR) were calculated for allele contrast and dominant inheritance models.

Results: In the case-control study, genotype and allele frequencies of the Asp299Gly and Thr399Ile polymorphisms differed between T2DM patients and nondiabetic subjects ($P < 0.05$). Moreover, the presence of the minor alleles of these polymorphisms were significantly associated with protection for T2DM, after adjusting for ethnicity, under a dominant model [Asp299Gly: OR=0.68 (95% CI 0.49-0.94); Thr399Ile: OR=0.65 (95% CI 0.46-0.90)]. Seven studies were eligible for inclusion in the meta-analysis. Meta-analysis results showed that the Asp299Gly polymorphism was associated with T2DM protection [OR=0.68 (95% CI 0.46-1.00), allele contrast model]. Stratification by ethnicity revealed that both polymorphisms were associated with T2DM protection under allele contrast and dominant models in Brazilian population but not in Europeans.

Conclusions: In our case-control study, we were able to demonstrate a possible association between the *TLR4* Asp299Gly and Thr399Ile polymorphisms and protection for T2DM. In agreement, the meta-analysis results showed an association of the

Asp299Gly polymorphism with T2DM protection in the whole group, and associations of the Asp299Gly and Thr399Ile polymorphisms with T2DM protection in the Brazilian group but not in European descendent. This is the largest *TLR4* meta-analysis performed so far. In other ethnicities further studies with large sample size are necessary to confirm these associations as well as to elucidate the roles possibly played by these polymorphisms in the pathogenesis of T2DM.

Keywords: Type 2 diabetes mellitus, Toll-like receptor 4, DNA polymorphisms, case-control study, meta-analysis.

Introduction

Type 2 diabetes mellitus (T2DM) is a chronic and complex disease caused by a combination of insulin resistance (IR) and impaired insulin secretion from pancreatic beta-cells [1]. According to the “metainflammation” (metabolically triggered inflammation) hypothesis, both T2DM and IR are considered as states of preclinical chronic low-grade inflammation [2], resulting from changes in the innate immunity response, which is the first line of defense against viruses, bacteria and fungi [3,4]. Therefore, polymorphisms in genes that encode proteins related to the innate immune system, such as the toll-like receptors (TLRs), could influence the immune response as well as the development of T2DM [5].

TLRs are evolutionary conserved pattern-recognition receptors (PRRs) that play a key role in the activation of innate immune response by recognizing highly conserved pathogen-associated molecular patterns (PAMPs), such as the lipopolysaccharide (LPS), component of gram-negative bacteria [4,6]. Human orthologs are known to comprise at least 10 members [7]. Each TLR family member recognizes a specific pathogen component and, upon activation, triggers a signaling cascade leading to the production of inflammatory cytokines, releasing of antimicrobial peptides, and activation of the adaptive immune response [8,9].

Toll-like receptor 4 (TLR4) recognizes LPS as a ligand [9], and is expressed in macrophages, airway epithelia, adipose tissue, skeletal muscle, pancreas, and vascular endothelial and smooth muscle cells [10]. It also interacts with endogenous ligands such as free-fatty acids, heat shock proteins 60 and 70, fibrinogen and fibronectin, which are elevated in T2DM patients [10-12]. Moreover, TLR4 activation seems to play a critical

role in the pathogenesis of IR and T2DM in both clinical and experimental conditions [10,13-15].

Two functional polymorphisms in the *TLR4* gene (Asp299Gly and Thr399Ile) were reported as being associated with LPS hyporesponsiveness [16]. Since their identification, these polymorphisms have been investigated for their association with T2DM and IR; however, results from such studies have been contradictory [5,17-22]. Considering that T2DM is caused by interactions between a large number of environmental and genetic factors, it is not unexpected that some genetic association studies will fail to confirm associations with the disease even if they actually exist. Indeed, T2DM has been described as a “geneticist’s nightmare” and it may be the case that it will be necessary to study huge numbers of patients in order to elucidate the associations between even a single polymorphism and the disease [23]. Therefore, as part of the ongoing effort to examine the hypothesis that *TLR4* polymorphisms are associated with T2DM risk, we performed a case-control study in a Brazilian population, followed by a meta-analysis of the literature on the subject.

Material and Methods

1. Case-control study

1.1 Population

The diabetic sample comprised 1684 T2DM patients participating in a multicenter study that began recruiting patients in Southern Brazil in 2002. That project was aimed to study risk factors for T2DM and its chronic complications. It initially included four centers in University hospitals located in the Brazilian state of Rio Grande do Sul: Grupo Hospitalar Conceição, Hospital São Vicente de Paula, Hospital

Universitário de Rio Grande, and Hospital de Clínicas de Porto Alegre. A detailed description of that study can be found elsewhere [24]. T2DM was diagnosed according to the American Diabetes Association criteria [1]. The main characteristics of the T2DM patients were as follows: mean age was 58.5 ± 10.4 years, mean T2DM duration was 12.3 ± 9.3 years, mean age at T2DM diagnosis was 46.3 ± 11.4 years, mean glycated hemoglobin (HbA1c) was $7.8 \pm 1.7\%$, and mean body mass index (BMI) was 28.9 ± 5.2 kg/m². Males comprised 47.4% of the sample and 72.2% of all patients had arterial hypertension (AH).

The nondiabetic group comprised 584 healthy volunteers attending the blood donation facility at Hospital de Clínicas de Porto Alegre (Porto Alegre, Brazil; males= 51.0%; mean age= 44.0 ± 7.8 years). None of these individuals had diabetes or a family history of it. The ethnic group was defined based on self-classification, and the ethnic proportion between the two samples was as follows: 20.1% of black patients in the T2DM group and 12.3% of black subjects in the nondiabetic group.

A standard questionnaire was used to collect information regarding age, age at T2DM diagnosis, and drug treatment. All T2DM patients underwent detailed physical and laboratory evaluations, as previously described [25,26]. Briefly, they were weighed bare feet, wearing light outdoor clothes and their height was measured. BMI was calculated as weight (kg)/height² (meters). Office blood pressure (BP) was measured in sitting position, on the left arm, after a 5-min rest by a trained researcher, with a mercury sphygmomanometer. The mean of two measurements taken 1 min apart was used to calculate both systolic and diastolic BP. Hypertension (AH) was defined as BP levels higher than 140/90 mmHg at the initial visit and at two follow-up visits within 1 month of the initial visit, or if the presence of AH was previously registered on medical records.

Serum and plasma samples from T2DM patients were taken after a 12 hours of fasting for laboratory analyses. Plasma glucose levels were determined using the glucose oxidase method. HbA1c measurements were performed by different methods and results were traceable to the Diabetes Control and Complications Trial (DCCT) method by off-line calibration or through conversion formulae [27].

Total plasma cholesterol, HDL cholesterol and triglycerides were assayed using enzymatic methods. Nondiabetic individuals did not undergo any of these tests.

The information obtained from this study did not influence patients' diagnosis or treatment. The study was approved by the Ethic Committee in Research from Hospital de Clínicas de Porto Alegre and all subjects provided written informed consent.

1.2 Genotyping

DNA was extracted from peripheral blood leucocytes by a standardized salting-out procedure [28]. *TLR4* Asp299Gly (rs4986790; A/G) and Thr399Ile (rs4986791; C/T) polymorphisms were genotyped using primers and probes contained in the 40x Human Custom TaqMan Genotyping Assay (Life Technologies, Foster City, CA). Sequences of primers and probes were: 5'–ACCATTGAAGAATTCCGATTAGCATACT–3' (forward); 5'–CACCAGGGAAAATGAAGAAACATTTGT–3' (reverse); 5'–CCTCGATGGTATTATT–3' (FAM dye); 5'–CTACCTCGATGATATTATT–3' (VIC dye) for the Asp299Gly polymorphism, and 5'–GAGTTTCAAAGGTTGCTGTTCTCAA–3' (forward); 5'–GGTAATAACACCATTGAAGCTCAGATCT–3' (reverse); 5'–TTAGGCTGATTGTCCC–3' (FAM dye); 5'–TTAGGCTGGTTGTCCC–3' (VIC dye) for the Thr399Ile polymorphism. Reactions were conducted in 384-well plates, in a total

of 5 μ L volume using 2 ng of genomic DNA, TaqMan Genotyping Master Mix 1x (Life Technologies) and Custom TaqMan Genotyping Assay 1x. Plates were then loaded in a real-time PCR thermal cycler (ViiA-7 Real time PCR System; Life Technologies) and heated for 10 min at 95°C, followed by 50 cycles of 95°C for 15s and 60°C for 1 min.

1.3 Statistical analyses for case-control study.

Allele frequencies were determined by gene counting and departures from the Hardy-Weinberg equilibrium (HWE) were analyzed using χ^2 tests. Allele and genotype frequencies were compared between groups using χ^2 tests. Clinical and laboratory characteristics were compared between genotype groups by using unpaired Student's t-test, One-way ANOVA or χ^2 , as appropriate. Variables with normal distribution are presented as mean \pm SD or percentage. Variables with skewed distribution were log-transformed before analyses and are represented as median (minimum – maximum values).

The magnitude of associations of the Asp299Gly and Thr399Ile polymorphisms with T2DM were estimated using OR (95% CI). Multivariate logistic regression analyses were performed to assess the independent association of each of the two analyzed polymorphisms with T2DM, adjusting for ethnicity and age. Results for which the P value was under 0.05 were considered statistically significant. Bonferroni correction was used to account for multiple comparisons. These statistical analyses were performed using SPSS version 18.0 (SPSS, Chicago, IL).

Between all pairs of biallelic loci, we examined widely used measures of linkage disequilibrium, Lewontin's D' $|D'|$ and r^2 [29]. The haplotype constructed from the combination of the two *TLR4* polymorphisms and their frequencies were inferred using the software Phase 2.1, which implements a Bayesian statistical approach. We also used

Phase 2.1 to compare the distributions of the different *TLR4* haplotypes between T2DM patients and nondiabetic subjects through permutation analysis of 10,000 random replicates [30].

Power calculations were done using the software PEPI, version 4.0, and showed that this study has a power of approximately 80% at a significance level of 0.05 to detect an OR of 0.65 or less (for the presence of the minor alleles).

2. Meta-analysis

2.1 Selection criteria and search strategy.

PubMed, Cochrane and Embase repositories were searched to identify all articles that analyzed associations between T2DM and at least one of the two polymorphisms of interest. The following medical subject headings (MeSH) were used for this search: (“diabetes mellitus, type 2” OR “diabetes mellitus”) AND (“polymorphism, genetic” OR “polymorphism, single-stranded conformational” OR “polymorphism, single nucleotide” OR “polymorphism, restriction fragment length” OR “amplified fragment length polymorphism analysis” OR “DNA copy number variations”) AND (“mutation” OR “frameshift mutation” OR “mutation rate” OR “INDEL mutation” OR “mutation, missense” OR “point mutation” OR “codon, nonsense”) AND (“toll-like receptor 4” OR “toll-like receptors”). The search was restricted to human studies and English or Spanish language articles and was completed on January 10, 2014. All of the articles identified were searched manually to identify any other relevant citations. This study was designed and described in agreement with widely used guidelines for execution of systematic reviews and meta-analyses [31,32].

2.2 Study selection and data extraction.

Eligibility evaluation was made by title and abstracts review and when abstracts did not provide sufficient data, the full text of the paper was retrieved for analysis. This was done independently in a standardized manner by two investigators (T.S.A and N.E.L). Disagreements were resolved by discussion between them and when required a third reviewer (D.C.) was consulted. Articles were excluded from the analysis if the genotype frequencies in the control group deviated from those predicted by the HWE and if they did not have enough data to estimate an OR with 95% CI. If data were duplicated and had been published more than once, the most complete study was chosen [25,26,33,34].

Necessary information from each study was independently extracted by two investigators (T.S.A. and N.E.L.) using a standardized extraction form, and consensus was sought in all extracted items. When consensus could not be achieved, differences in data extraction were decided by reading the original publication. The data extracted from each study was as follows: (1) characteristics of study participants (including name of first author, publication year, number of subjects in case and control groups, mean age, gender, BMI, ethnicity, age at diagnosis, fasting plasma glucose, total cholesterol, HDL cholesterol, LDL cholesterol, systolic BP, diastolic BP); (2) case and control definitions; (3) polymorphism frequencies [including genotype and allele distributions in case and control groups and OR (95% CI)].

2.3 Quality assessment

To ascertain the validity of each included study, two investigators (T.S.A and N.E.L.) independently assessed the quality using the Newcastle-Ottawa Scale (NOS) [32]. The NOS contains eight items divided into three dimensions: selection, comparability, and exposure. For each item, a sequence of answer options is provided.

A star scoring system is used to allow a semi-quantitative evaluation of paper quality, such that the highest quality studies are given a maximum of one star for each item, with exception of the item related to comparability, which allows two stars to be given. Therefore, the total NOS score can vary from zero to nine stars.

2.4 Statistical analysis for meta-analysis.

Control subjects' genotype distributions were tested for conformity with HWE using a goodness-of-fitness χ^2 test. Gene-disease associations were measured using OR (95% CI) estimation based on allele contrast and dominant model of inheritance [35,36]. Other inheritance models were not analyzed due to the rarity of the minor alleles. Heterogeneity was tested using χ^2 -based Cochran's Q statistic and inconsistency was assessed with the I^2 metric, as previously described [25,26,33,34]. Briefly, heterogeneity was considered statistically significant at $P < 0.10$ for the Q statistic and $I^2 > 50\%$ for the I^2 metric statistics. Where significant heterogeneity was detected, the DerSimonian and Laird random effect model (REM) was used to calculate OR (95% CI) for each study and for the pooled effect; where heterogeneity was not significant, the fixed effect model (FEM) was used for this evaluation [37]. Meta-regression and sensitivity analyses were performed to identify important studies with a significant impact on inter-study heterogeneity [25,26,33,34]. The factors investigated by meta-regression were age, BMI and gender. Sensitivity analyses were done after stratifying the studies by ethnicity, given that the *TLR4* polymorphisms show variable distributions across different ethnic groups. Risk of publication bias was evaluated using funnel plot graphics, analyzed both visually and by using the Begg and Egger test [38]. $P < 0.10$ was considered indicative of statistically significant publication bias. All statistical analyses were performed using Stata 11.0 software (StataCorp, College Station, TX, USA).

Results

Case-control study

Genotype and allele frequencies of the *TLR4* Asp299Gly and Thr399Ile polymorphisms in T2DM patients and nondiabetic subjects are depicted in **Table 1**. The frequency of the G allele of the Asp299Gly polymorphism was 0.05 in white subjects and 0.04 in black subjects ($P= 0.238$), while the frequency of the T allele of the Thr399Ile polymorphism was 0.046 in white and 0.047 in black subjects ($P= 0.920$). All genotypes were in agreement with those predicted by the HWE ($P >0.05$). Both genotype and allele frequencies of the two analyzed polymorphisms were differently distributed between T2DM patients and nondiabetic subjects after Bonferroni corrections (**Table 1**). Genotype frequencies of both polymorphisms remained significantly associated with T2DM after adjustment for ethnicity and age (**Table 1**). In agreement with this data, the presence of the minor alleles of the Asp299Gly and Thr399Ile polymorphisms were significantly associated with protection for T2DM under a dominant model of inheritance, adjusting for ethnicity and age [OR = 0.68 (95% CI 0.49-0.94) and OR = 0.65 (95% CI 0.46-0.90), respectively; **Table 1**]. Of note, after the exclusion of black patients from the sample, the presence of the minor alleles was still associated with protection for T2DM in white subjects under a dominant model of inheritance [Asp299Gly: OR = 0.61 (95% CI 0.43-0.86); Thr399Ile: OR = 0.64 (95% CI 0.45-0.91)].

The frequencies of the different haplotypes produced by the combination of the *TLR4* Asp299Gly and Thr399Ile polymorphisms in T2DM patients and nondiabetic subjects are shown in **Table 2**. Four haplotypes were inferred in both groups of subjects, and the distributions of these haplotypes were statistically different between

T2DM patients and nondiabetic subjects ($P = 0.023$). Differences in the frequencies of the two most common haplotypes (A-C and G-T), which had opposite alleles for the two studied positions, explain this observation. Interestingly, the prevalence of T2DM was lower as more minor alleles were present in the subjects (P -trend = 0.022) (**Table 2**). The presence of four minor alleles was associated with the highest protection for T2DM [OR = 0.21 (95% CI 0.05-0.86)].

The *TLR4* Asp299Gly and Thr399Ile polymorphisms are in moderate linkage disequilibrium ($|D'| = 0.85$; $r^2 = 0.73$) in our samples. For that reason, in **Table 3**, we present only clinical and laboratorial characteristics of T2DM patients according to the presence of the minor allele of the Asp299Gly polymorphism. Gender, age, age at T2DM diagnosis, HbA1c, systolic and diastolic BP, and lipid profile did not differ between patients categorized by the presence of the 299Gly allele after Bonferroni corrections. Similar results were found for the Thr399Ile polymorphism (data not shown).

Meta-analysis

One-hundred seventy-four possible relevant citations were retrieved by searching the electronic databases, and 146 of them were excluded following the reading of titles and abstracts. Twenty-eight articles seemed to be eligible after this phase and, then, their full texts were evaluated. Nevertheless, after cautious analysis of the full texts, another 22 papers were excluded owing to missing information, ineligible study designs or because they investigated other *TLR4* polymorphisms. Eleven articles fulfilled the eligibility criteria; however, only 7 were included in the meta-analysis: 6 that had been identified through the database searches [5,17-21], in addition to the case-control study we described above (**Figure S1**). The other four studies evaluated the

Asp299Gly and Thr399Ile polymorphisms in Asian populations, but they could not be included in the meta-analysis since only the ancestral genotypes were found [39-42].

Clinical characteristics for each individual paper are described in **Table S1**. All of these studies analyzed the Asp299Gly polymorphism (3583 cases / 3813 controls), while 6 studies investigated the Thr399Ile polymorphism (3262 cases / 1846 controls). **Table S2** lists genotype and allele distributions and ORs (95% CI) for the *TLR4* polymorphisms in case and control groups from the different studies reviewed. The quality of each study is shown in **Table S3**. The highest quality studies were awarded nine stars. In general, most studies were considered as having good quality selection, comparability and exposure. None of the papers scored less than six stars and 80% of them had seven or eight stars.

Table 4 summarizes the results of the pooled analyses for associations between *TLR4* Asp299Gly and Thr399Ile polymorphisms and susceptibility to T2DM. The Asp299Gly polymorphism was associated with T2DM protection when assuming an allele contrast model (REM OR 0.68, 95% CI 0.46-1.00). Furthermore, stratification by ethnicity showed that, assuming an allele contrast model, the 299Gly allele was significantly associated with protection for T2DM in Brazilian population [OR = 0.59 (95% CI 0.45-0.79), but not in Europeans (**Figure 1A**). When assuming the dominant model, the 299Gly allele was associated with T2DM protection in Brazilians [0.62 (95% CI 0.47-0.82)], but not in Europeans or in the whole sample (**Table 4**). In the whole sample the Thr399Ile polymorphism was not associated with T2DM under allele contrast or dominant models (**Table 4**). However, stratification by ethnicity showed that the 399Ile allele was associated with T2DM protection in Brazilians under allele contrast [OR = 0.58 (95% CI 0.44-0.76); **Figure 1B**] and dominant [OR = 0.60 (95% CI 0.45-0.81)] models, whereas it was not associated with the disease in Europeans for the

two analyzed models (**Table 4**). There was only one study performed in Mexicans, showing that the two analyzed polymorphisms were not associated with T2DM in this population (**Figure 1**).

There was significant heterogeneity among studies investigating the *TRL4* polymorphisms when assuming allele contrast and dominant models ($I^2 > 50\%$ for the entire sample), as it can be observed in **Table 4**. To investigate this result in more details, gender, age and BMI were used as covariates in the meta-regression analyses performed for the two *TLR4* polymorphisms. None of the covariates used in univariate meta-regression analyses could independently explain the heterogeneity observed for the allele contrast and dominant models (data not shown).

Sensitivity analyses were performed with the aim of estimating the influence of each study on the meta-analysis data. This was done by repeating the meta-analysis, but omitting a given study each time. The results showed that one paper [19], was responsible for the heterogeneity observed in the meta-analyses of Asp299Gly and Thr399Ile polymorphisms under dominant models. Thus, significant heterogeneity was abolished by exclusion of this study from the analysis regarding this inheritance model (**Table 4**). However, the exclusion of this study only changed significantly the result obtained for the Asp299Gly polymorphism [OR 0.68 (95% CI 0.48-0.96)]. Heterogeneity observed for allele contrast models could not be eliminated by the exclusion of any study. At last, no significant publication bias was identified for allele contrast and dominant models in the total sample (**Figures S2 and S3**).

Discussion

According to previously published evidence, TLR4 activation in adipocytes, skeletal muscles and liver induces up-regulation of proinflammatory pathways related to

IR and, consequently, T2DM [13,14,43,44]. Furthermore, a recent study showed that treatment with LPS inhibits insulin gene expression in rat and human pancreatic beta-cells in a TLR4-dependent manner and via NF- κ B signaling [45]. Importantly, the effects of LPS on the insulin gene in human islets were observed at concentrations similar to the circulating levels achieved during endotoxemia, suggesting that direct repression of the insulin gene might contribute to the metabolic disturbances associated with alterations of microbiota [45].

In agreement with this evidence, some studies have reported that the *TLR4* Asp299Gly and Thr399Ile polymorphisms are associated with T2DM [5,10,46,47]; however, results have been inconsistent. The contradictory results could be partially explained by the fact that the associations between these polymorphisms and T2DM are influenced by ethnic backgrounds [48]. Another source of confusion could come from independent analysis of these polymorphisms without considering that they are in tight linkage disequilibrium [49]. Moreover, the inconsistencies might be explained by studies with small sample sizes, lacking enough statistical power. Thus, in an attempt to arrive at a more definitive conclusion about the associations between these *TLR4* polymorphisms and T2DM, we performed a case-control study followed by a meta-analysis of genetic association studies on this subject.

Our case-control study showed that the presences of the minor alleles of *TLR4* Asp299Gly and Thr399Ile polymorphisms are associated with protection for T2DM in a South Brazilian population. It is worth mentioning that the frequencies of these polymorphisms in our samples were similar to those frequencies observed in another study performed in a Southeast Brazilian population [5]. Bagarolli *et al.* [5] also reported an association of these two polymorphisms with protection for T2DM.

Seven studies with 3904 T2DM patients and 4348 controls were included in our meta-analysis of the Asp299Gly polymorphism, and 6 studies (3581 cases and 2384 controls) were included in the meta-analysis of the Thr399Ile polymorphism. The meta-analysis results showed an association between the minor allele of the Asp299Gly polymorphism and protection for T2DM in the whole sample [OR = 0.68 (95% CI 0.46-1.00); allele contrast model]. In contrast, the Thr399Ile polymorphism was not associated with the disease in the whole sample. After stratification by ethnicity, the minor alleles of the Asp299Gly and Thr399Ile polymorphisms were significantly associated with protection for T2DM in Brazilian populations, but not in European descendants. Moreover, only one study evaluated these polymorphisms in Mexicans, and it did not show any association with T2DM [20].

Heterogeneity is potentially a significant problem when interpreting the data of meta-analyses of genetic association studies, and the present meta-analysis showed significant inter-study heterogeneity for both polymorphisms analyzed in the total group under allele contrast and dominant inheritance models. To examine this in greater deepness, sensitivity analyses were performed repeating the meta-analysis omitting a given study each time. The exclusion of the data from Buraczynska *et al.* [19] significantly decreased the heterogeneity observed in the meta-analyses of the Asp299Gly polymorphism under a dominant model. Moreover, after the exclusion of this study, the presence of the 299Gly allele was associated with T2DM protection [OR 0.68 (95% CI 0.48-0.96)]. Meta-regression analyses were not able to explain the heterogeneity observed for the whole group. This heterogeneity could be explained by differences in genotyping methods, sample selection or gene-environment interactions. Therefore, without full information on the metabolic and clinical data of the studies

included here, we could not completely eliminate the possibility that the heterogeneity observed might decrease our power to detect true associations.

It is noteworthy that one previous meta-analysis investigated the association between the *TLR4* Asp299Gly polymorphism and metabolic disorders, and it included 6 studies totalizing 1696 cases with T2DM and/or metabolic syndrome and 3388 controls [22]. The authors reported a significant association between the 299Gly allele and protection for metabolic disorders [OR = 0.57 (95% CI 0.35-0.93)], which is in agreement with our data. However, after stratification by ethnicity, this association was maintained in Caucasians [OR = 0.50 (95% CI 0.28-0.99)], but not in samples having mixed ethnicity, which conflicts with our data showing an association only in Brazilians. We believe that a possible reason for this discrepancy is that the meta-analysis of Belforte *et al.* [22] included one study [50] where cases were patients with metabolic syndrome instead of patients with T2DM. They did not inform the number of patients with metabolic syndrome that also had T2DM. The inclusion of a study that analyzed a different outcome (metabolic syndrome) could have increased the heterogeneity of their data and, consequently, changed the results. Furthermore, they did not include the study of Buraczynska *et al.* [19] from 2009, which shows no association between the Asp299Gly polymorphism and T2DM.

It is supposed that the ability to respond efficiently to different ligands might be impaired by polymorphisms in the *TLR4* gene [20]. In this context, the mutated alleles of the Asp299Gly and Thr399Ile polymorphisms have been shown to change the ligand-binding site of the TLR4, often resulting in hyporesponsiveness of the immune system [16]. Recently, Figueroa *et al.* [48] reported that the 299Gly allele causes a deficient recruitment of the adapters MyD88 and TRIF to the TLR4, consequently leading to a LPS-hyporesponsive phenotype. In agreement with these data, individuals carrying the

299Gly allele have lower levels of proinflammatory cytokines, acute-phase reactants, and soluble adhesion molecules, such as interleukin 6 and fibrinogen [51]. Moreover, Asp299Gly and Thr399Ile polymorphisms have been reported to be associated with different diseases such as gastric cancer, hepatitis C virus-induced hepatocellular carcinoma, Crohn's Disease and ulcerative colitis [16,52,53], supporting a functional role for these polymorphisms. Also, these two polymorphisms seem to modify the pattern of inflammatory cytokines and chemokines in the gastric mucosa, suggesting a contribution to the clinical outcome of *H. pylori* infection [54].

Our literature search identified four studies that evaluated the Asp299Gly and Thr399Ile polymorphisms in Asian populations, but they could not be included in the present meta-analysis because only the ancestral genotypes were found [39-42]. The lower frequency of the minor alleles of these *TLR4* polymorphisms in Asians will require larger sample sizes to detect any statistically significant association with T2DM. Recently, the *TLR4* rs11536889 (+3725G/C) polymorphism was reported as being associated with protection for T2DM in a Chinese population [42]. This polymorphism is located at the 3' untranslated region of the gene and seems to induce a lower rate of *TLR4* mRNA translation [55]. Further studies are necessary to evaluate the association between the *TLR4* rs11536889 and T2DM in other populations.

Some factors could have interfered with the findings of our case-control study. First, we cannot rule out the possibility of population stratification bias when analyzing our samples, even though both T2DM patients and non-diabetic subjects were recruited from the same hospital, the results were adjusted for ethnicity, and the exclusion of black subjects from the whole sample did not change the association observed, thus reducing the risk of false-positive/negative associations due to this bias. Second, we did not perform a replication of the observed association in another Brazilian sample,

although our data is in agreement with a previous study performed in Southeast Brazilian subjects [5]. In the same way, the results of our meta-analysis should be interpreted within the context of a few limitations. First, meta-analysis is prone to publication bias, and although we have attempted to trace unpublished studies, we cannot be sure that small negative studies were overlooked. Moreover, we did not detect any significant publication bias in our analysis, showing that our data is statistically reliable. Second, because of the difficulty in getting the full texts of articles published in several languages, we only included studies published in English and Spanish. Third, inter-studies heterogeneity is common problem in meta-analyses of genetic association studies [56], and our meta-analyses showed significant inter-study heterogeneity for both *TLR4* polymorphisms analyzed. Although meta-regression analyses were not able to explain this heterogeneity, sensitivity analyses did not change significantly the reported associations.

In conclusion, our results indicate that the *TLR4* Asp299Gly and Thr399Ile polymorphisms are associated with protection for T2DM in Brazilians. The meta-analysis results showed an association of the Asp299Gly polymorphism with T2DM protection in the whole group, and associations of the Asp299Gly and Thr399Ile polymorphisms with T2DM protection in the Brazilian group but not in European descendent. This is the largest *TLR4* meta-analysis performed so far. In other ethnicities further studies with large sample size are necessary to confirm these associations in different ethnicities as well as to elucidate the roles possibly played by these polymorphisms in the pathogenesis of T2DM. To this date, *TLR4* activation seems to play a critical role in the pathogenesis of IR and T2DM in both clinical and experimental conditions [10,13-15]. Thus, as suggested by Reina *et al.* [14], future

strategies aimed at reducing *TLR4* expression, or at blocking TLR4 signaling may prove useful in enhancing insulin sensitivity in subjects with IR.

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Table 1. Genotype and allele distributions of *TLR4* Asp299Gly and Thr399Ile polymorphisms in T2DM patients and nondiabetic subjects.

<i>TLR4</i> polymorphisms	T2DM patients	Nondiabetic subjects	Unadjusted *P value	Adjusted OR (95% CI) / §P value
Asp299Gly (A/G)	n = 1672	n = 570		
A/A	1531 (91.6)	499 (87.5)	0.005	1
A/G	138 (8.2)	66 (11.6)		0.68 (0.5-0.93) / 0.015
G/G	3 (0.2)	5 (0.9)		0.20 (0.05-0.82) / 0.026
A	0.96	0.93	0.002	
G	0.04	0.07		
Dominant Model				
A/A	1531 (91.6)	499 (87.5)	0.006	1
A/G + G/G	141 (8.4)	71 (12.5)		0.68 (0.49-0.94) / 0.019
Thr399Ile (C/T)	n = 1684	n = 584		
C/C	1550 (92.0)	514 (88.0)	0.004	1
C/T	131 (7.8)	65 (11.1)		0.67 (0.49 – 0.92) / 0.012
T/T	3 (0.2)	5 (0.9)		0.20 (0.05 – 0.84) / 0.027
C	0.96	0.94	0.001	
T	0.04	0.06		

Dominant Model				
C/C	1550 (92.0)	514 (88.0)	0.004	1
C/T + T/T	134 (8.0)	70 (12.0)		0.65 (0.46-0.90) / 0.009

Data are presented as number of carriers (%) or proportion. *P values were computed using χ^2 tests to compare T2DM patients and nondiabetic subjects. § P values adjusted for ethnicity in a logistic regression analysis.

Table 2. *TLR4* gene haplotypes in T2DM patients and nondiabetic subjects.

	T2DM patients (n = 3274)	Nondiabetic subjects (n = 1124)	P* value
A-C	0.952	0.937	0.023
A-T	0.006	0.009	
G-C	0.011	0.011	
G-T	0.031	0.052	

n = number of chromosomes. The first letter of the haplotype refers to Asp299Gly (A/G) and the second to Thr399Ile (C/T). * P values for the comparisons of haplotypic frequencies between T2DM patients and nondiabetic subjects were calculated using permutation tests (10,000 replications).

Table 3. Clinical and laboratory characteristics of T2DM patients, broken down by the presence of the G allele of the *TLR4* Asp299Gly polymorphism (dominant model).

	A/A	A/G + G/G	P* value
Age (years)	56.1 ± 11.3	59.0 ± 11.8	0.007
Gender (% males)	49.3	51.9	0.592
Ethnicity (% black)	22.7	16.6	0.076
Age at diagnosis (years)	46.2 ± 11.3	47.6 ± 12.4	0.284
BMI (kg/m ²)	28.9 ± 5.2	28.6 ± 5	0.594
HbA1c (%; mmol/L)	7.2 ± 2.2 (55 ± 17)	6.8 ± 2.1 (51 ± 16)	0.166
Systolic BP (mmHg)	142.2 ± 24.1	143.3 ± 24.8	0.653
Diastolic BP (mmHg)	85.8 ± 14.3	84.6 ± 12.6	0.407
Total cholesterol (mmol/L)	5.3 ± 1.3	5.4 ± 1.2	0.557
HDL cholesterol (mmol/L)	2.2 ± 0.6	2.4 ± 1.1	0.250
Triglycerides (mmol/L)	3.6 (1.1 – 18.6)	3.3 (1.5 – 7.7)	0.836
Diabetic nephropathy (%)	52.2	51.2	0.916
Diabetic retinopathy (%)	49.6	49.4	0.999

Data are mean ± SD, median (minimum-maximum values) or %. BMI, body mass index; BP, blood pressure; HbA1c, glycated hemoglobin; T2DM, type 2 diabetes mellitus. *P values are according to χ^2 test or t-test as appropriate. Only P values lower than the Bonferroni threshold (P = 0.0038) were considered statistically significant.

Table 4. Meta-analysis pooled measures for associations between the *TLR4* Asp299Gly and Thr399Ile polymorphisms and the susceptibility to T2DM.

Inheritance model	n studies	n cases	n controls	I ² (%)	Pooled OR (95% CI)	Sensibility analysis*	
						I ² (%)	Pooled OR (95% CI)
Asp299Gly							
<i>Allele contrast</i>	7	3904	4348	81.5	0.68 (0.46 – 1.00)	77.2	0.60 (0.41 – 0.88)
European descendent	4	1700	3043	87.5	0.64 (0.34 – 1.22)		
Brazilians	2	1883	770	4.4	0.59 (0.45 – 0.79)		
Mexicans	1	321	535	-	1.35 (0.78 – 2.32)		
<i>Dominant</i>	5	3285	2374	64.6	0.89 (0.6 – 1.30)	27.9	0.68 (0.48 – 0.96)
European descendent	2	1081	1069	0.0	1.16 (0.80 – 1.68)		
Brazilians	2	1883	770	0.0	0.62 (0.47 – 0.82)		
Mexicans	1	321	535	64.6	1.36 (0.78 – 2.37)		
Thr399Ile							
<i>Allele contrast</i>	6	3581	2384	81.5	0.65 (0.39 – 1.07)	79.3	0.56 (0.32 – 0.97)
European descendent	3	1367	1062	90.5	0.65 (0.24 – 1.79)		
Brazilians	2	1895	784	54.3	0.46 (0.19 – 1.12)		
Mexicans	1	319	538	-	1.13 (0.64 – 2.00)		
<i>Dominant</i>	5	3295	1971	59.7	0.85 (0.58 – 1.26)	49.6	0.66 (0.40 – 1.10)
European descendent	2	1081	649	0.0	1.14 (0.78 – 1.65)		

Brazilians	2	1895	784	50.0	0.60 (0.45 – 0.81)
Mexicans	1	319	538	-	1.13 (0.63 – 2.03)

Where significant heterogeneity was detected ($I^2 > 50\%$), the DerSimonian and Laird random effect model (REM) was used to calculate OR (95% CI) for each individual study and for the pooled effect; where heterogeneity was not significant, the fixed effect model (FEM) was used for this calculation. * Sensibility analysis excluding the Buraczynska *et al* (Buraczynska et al., 2009) or to eliminate the heterogeneity.

Figure 1: **A)** Forest plots showing individual and pooled ORs (95% CI) for the associations between the *TLR4* Asp299Gly polymorphism and T2DM under an allele contrast inheritance model. The areas of the squares reflect the weight of each individual study and the diamonds illustrate the random-effects summary ORs (95% CI). **B)** Forest plots showing individual and pooled ORs (95% CI) for the associations between the *TLR4* Thr399Ile polymorphism and T2DM under an allele contrast inheritance model. The areas of the squares reflect the weight of each individual study and the diamonds illustrate the random-effects summary ORs (95% CI).

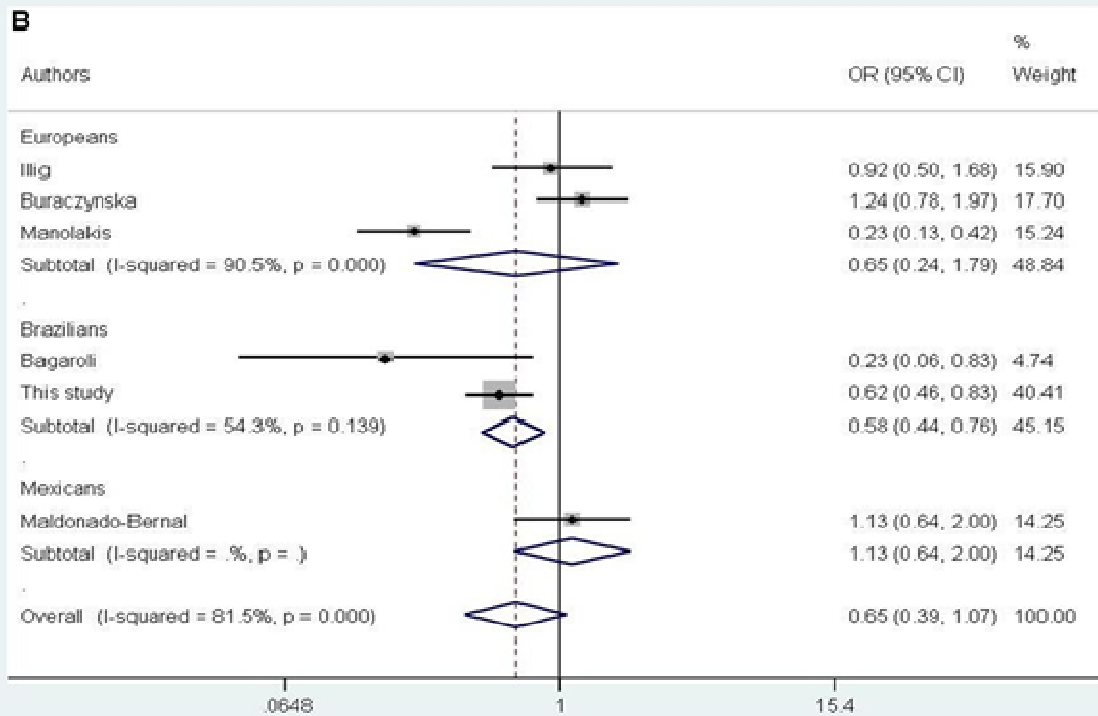
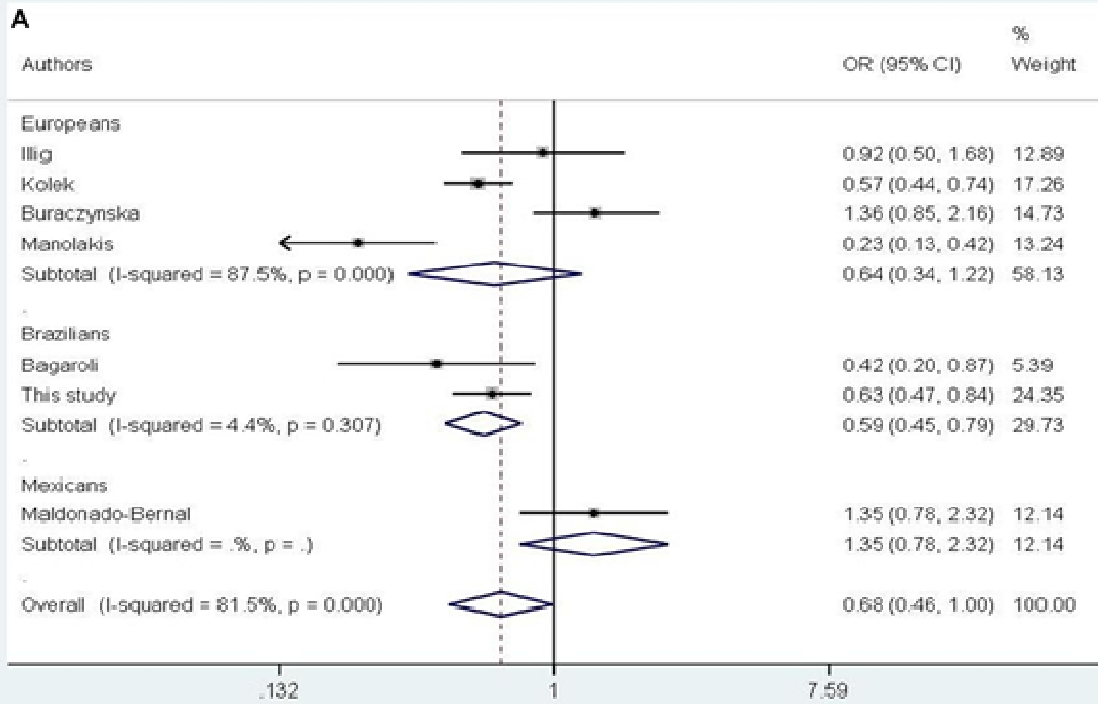


Figure S1 Flow diagram illustrating the strategy used to identify and select studies for inclusion in the meta-analysis.

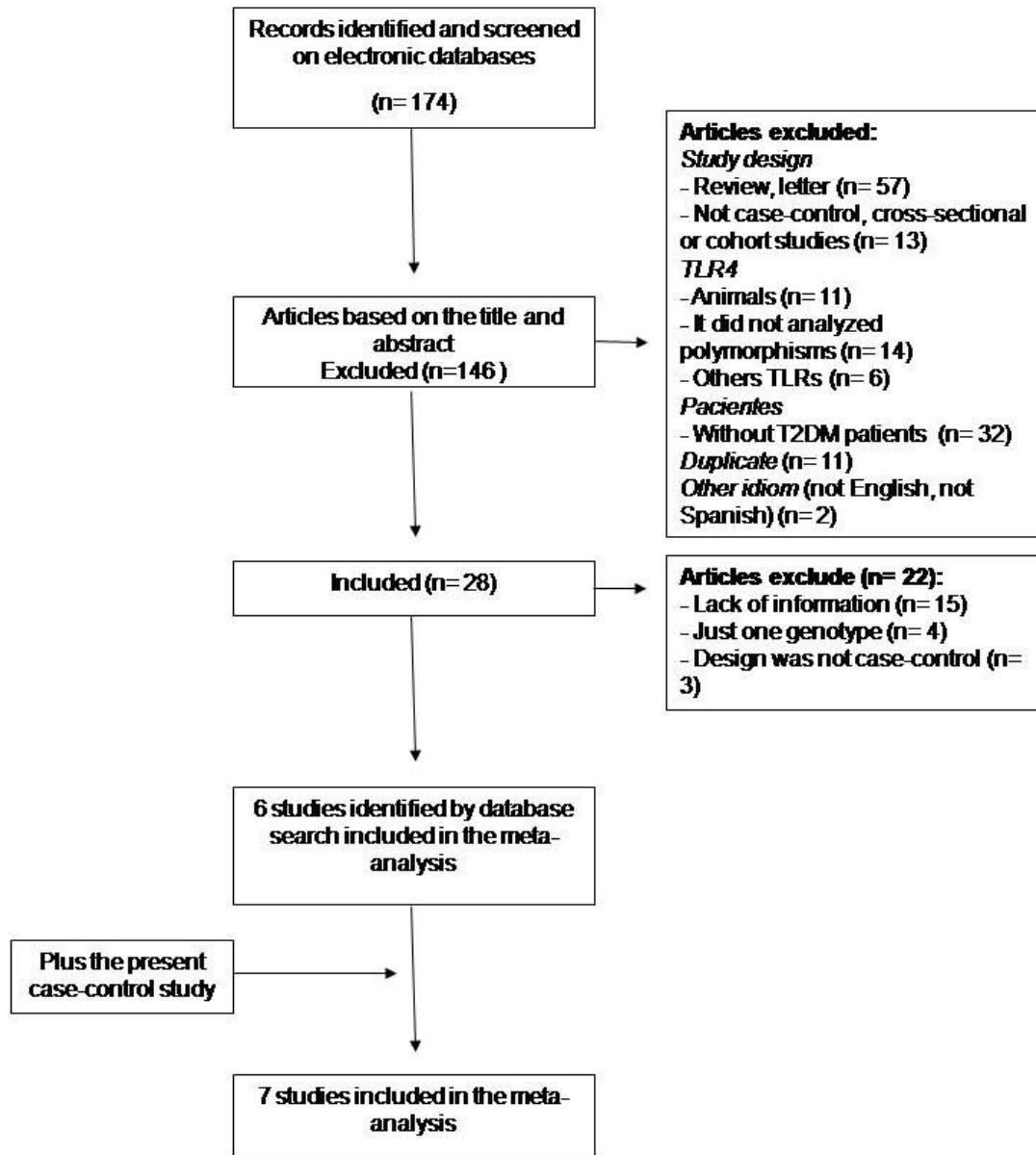


Figure S2 Funnel plot for contrast allele model for *TLR4* Asp299Gly polymorphisms.

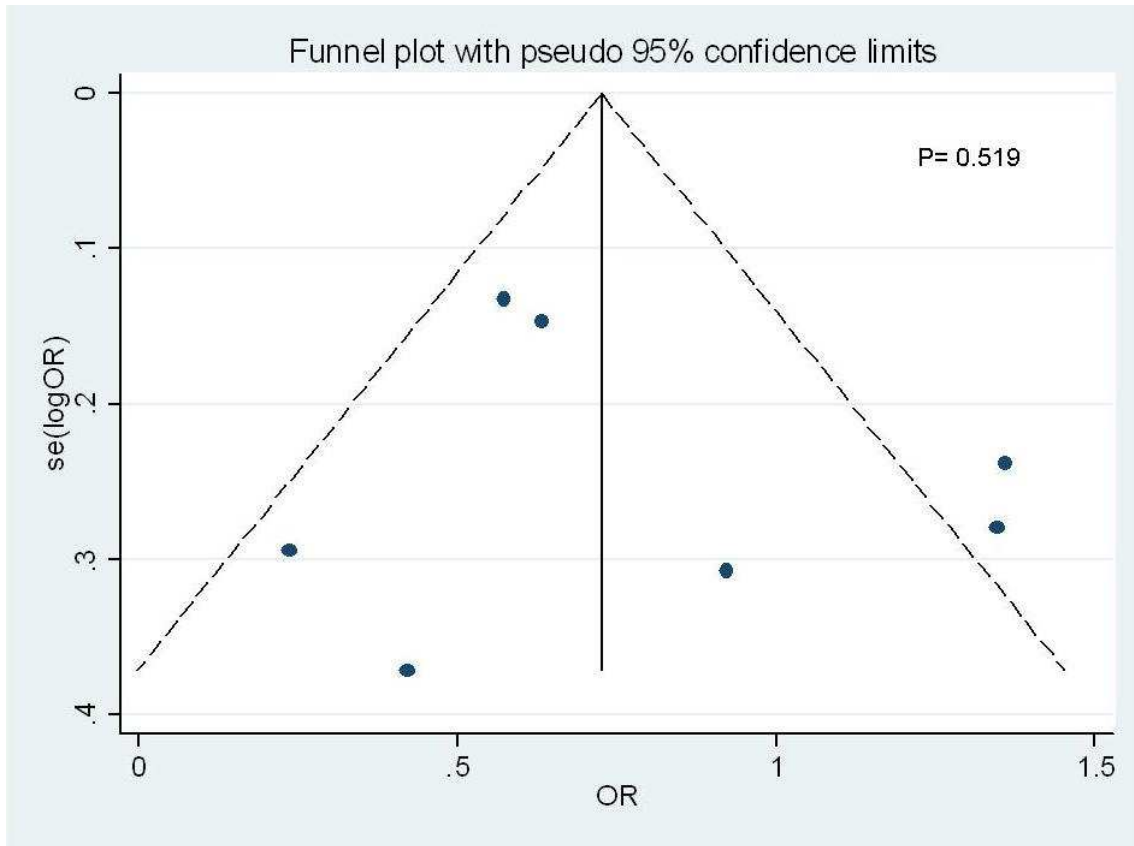


Figure S3 Funnel plot for contrast allele model for *TLR4* Thr399Ile polymorphisms.

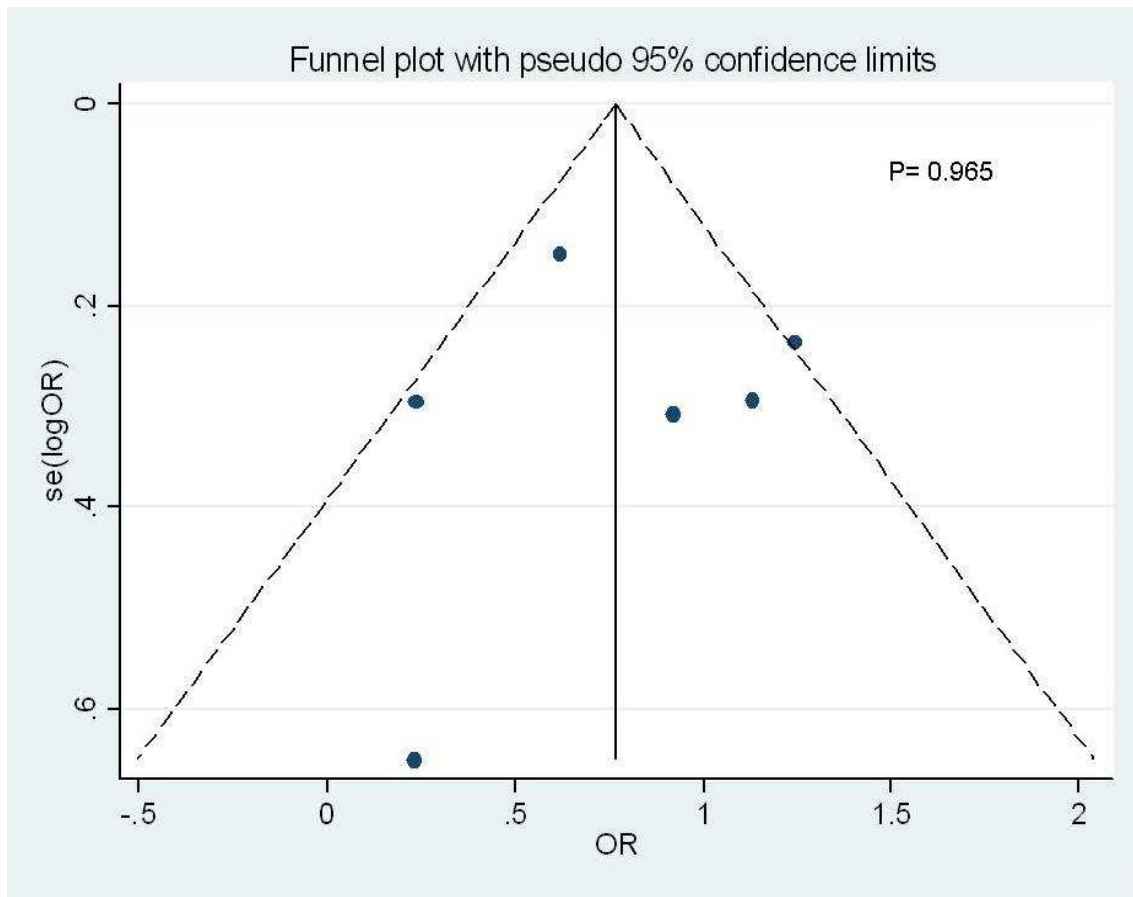


Table S1. Characteristics of the eligible studies in the meta-analysis.

Author (Ref.)	Year	Country	Ethnicity	Polymorphism	Age (years)	Male (%)	BMI (kg/m²)
Illig et al	2003	Germany	European	Asp299Gly; Thr399Ile	65.1 ± 5.2	59.4	ND
Kolek et al	2004	U.S.A	European	Asp299Gly	64.0 ± 11.0	69.0	28.5 ± 6.0
Buraczynska et al	2009	Poland	European	Asp299Gly; Thr399Ile	56.2 ± ND	53.5	26.7 ± 4.6
Bagaroli et al	2010	Brazil	Mixed	Asp299Gly; Thr399Ile	59.0 ± ND	54.1	31.8 ± ND
Manolakis et al	2011	Greece	European	Asp299Gly; Thr399Ile	62.3 ± 5.7	82.0	25.8 ± 3.5
Maldonado-Bernal et al	2011	Mexico	Mixed	Asp299Gly; Thr399Ile	43.6 ± 6.4	74.5	27.4 ± 3.5
Present case-control study	2013	Brazil	Mixed	Asp299Gly; Thr399Ile	56.2 ± 11.3	47.4	28.9 ± 5.2

Data are shown as mean ± SD or %; ND, no data.

Table S2 . Genotype and allele distributions of the *TLR4* Asp299Gly and *TLR4* Thr399Ile polymorphisms in patients with type 2 diabetes mellitus (cases) and non-diabetic subjects (controls)

<i>TLR4</i> Asp299Gly (A/G)			Cases (n) by total and genotype				Controls (n) by total and genotype				G allele frequency (%)		
Reference	Year	Ethnicity	Total	A/A	A/G	G/G	Total	A/A	A/G	G/G	Cases	Controls	OR (95% CI) ^a
Illig <i>et al</i>	2003	Europeans	217	196	21	0	229	207	20	2	4.8	5.2	0.919 (0.504 – 1.677)
Kolek <i>et al</i>	2004	Europeans	333	ND	ND	ND	1561	ND	ND	ND	11	18	0.569 (0.395 – 0.821)
Buraczynska <i>et al</i>	2009	Europeans	864	796	64	4	840	393	27	0	4.1	3.2	1.309 (0.834 – 2.053)
Bagaroli <i>et al</i>	2010	Mixed	211	200	11	0	200	178	20	2	2.6	6.0	0.419 (0.202 – 0.867)
Manolakis <i>et al</i>	2011	Europeans	286	ND	ND	ND	413	ND	ND	ND	5.5	23.6	0.234 (0.155 – 0.351)
Maldonado- Bernal <i>et al</i>	2011	Mixed	321	297	24	0	535	505	30	0	4.0	3.0	1.346 (0.779 – 2.324)
The present case-control study	2013	Mixed	1672	1531	138	3	570	499	66	5	4.3	6.7	0.63 (0.473 – 0.838)

<i>TLR4</i> Thr399Ile (C/T)			Cases (n) by total and genotype				Controls (n) by total and genotype				T allele frequency (%)		
Reference	Year	Ethnicity	Total	C/C	C/T	T/T	Total	C/C	C/T	T/T	Cases	Controls	OR (95% CI) ^a
Illig <i>et al</i>	2003	Europeans	217	196	21	0	229	207	20	2	4.8	5.2	0.919 (0.504 – 1.677)
Buraczynska <i>et al</i>	2009	Europeans	864	800	62	2	420	394	26	0	3.8	3.1	1.243 (0.783 – 1.972)
Bagaroli <i>et al</i>	2010	Mixed	211	208	3	0	200	189	10	1	0.7	3.0	0.231 (0.064 – 0.826)
Manolakis <i>et al</i>	2011	Europeans	286	ND	ND	ND	413	ND	ND	ND	5.5	23.6	0.234 (0.155 – 0.351)
Maldonado- Bernal <i>et al</i>	2011	Mixed	319	299	20	0	538	508	30	0	3.1	2.7	1.128 (0.635 – 2.004)
The present case-control study	2013	Mixed	1684	1550	131	3	584	514	65	5	4.1	6.4	0.617 (0.462 – 0.825)

^a Calculated from the reported genotypes

Table S3. Newcastle-Ottawa quality assessment scale for the studies included in the meta-analysis

Author [Ref.]	Year	Selection	Comparability	Exposure
Illig <i>et al</i>	2003	***	**	**
Kolek <i>et al</i>	2004	**	**	**
Buraczynska <i>et al</i>	2009	****	**	**
Bagaroli <i>et al</i>	2010	***	**	**
Manolakis <i>et al</i>	2011	***	**	**
Maldonado-Bernal <i>et al</i>	2011	****	**	**
This study	2013	****	**	**

CONCLUSÕES GERAIS

Em nosso estudo de caso-controle, demonstramos uma possível associação entre os polimorfismos Asp299Gly e Thr399Ile no gene *TLR4* e proteção para DM2. Da mesma forma, os resultados da meta-análise mostraram uma associação do polimorfismo Asp299Gly com proteção para o DM2 em todos os grupos étnicos. No entanto, após estratificação por etnia, encontramos associação entre os dois polimorfismos estudados e proteção para o DM2 em brasileiros, mas não em descendentes de europeus. Essa é a maior meta-análise que investigou os polimorfismos Asp299Gly e Thr399Ile no gene *TLR4* até o presente momento. Em outras etnias são necessários novos estudos com amostras maiores para confirmar essas associações, bem como elucidar os papéis possivelmente desempenhados por esses polimorfismos na patogênese do DM2.

Embora mais estudos sejam necessários para avaliar o efeito dos polimorfismos Asp299Gly e Thr399Ile no gene *TLR4* na patogênese do DM2, hipotetizamos que a exposição a algumas gorduras presentes na dieta, faz com que o TLR4 desencadeie processos inflamatórios, estimulando citocinas próinflamatórias em macrófagos e nos tecidos, que conseqüentemente levam a resistência à insulina no músculo e em outros tecidos. Juntamente a isso, o nosso estudo sugere que na presença dos alelos mutados dos polimorfismos aqui estudados, haja uma proteção ao desenvolvimento do DM2, evitando com que o reconhecimento de gorduras como sendo organismos não próprios ocorra.

ANEXO I

Comentários dos revisores da revista *Journal of Diabetes & Metabolism*

Reviewer #1: The study is well conducted with appropriate methodology and the results were well presented and consistent with the objectives of the study. The criticism with the current manuscript is that the limitations and strengths of the study were not discussed. Besides, why there are more cases than controls?

Answer: Thank you for the suggestion. We now included a paragraph of limitations in the discussion section of the article (page 19, last paragraph). Unfortunately, we have more cases than controls because samples from subjects for the nondiabetic group are still being collected in the blood donation facility of Hospital de Clínicas de Porto Alegre. Taking into account that having the same number of control and case subjects would take a long time, we decided to finish the study with the present number of subjects. We are aware that having 3 times more controls than cases could have interfered with our results. However, it is worth noting that we re-analyzed our data using only the first 500 T2DM patients included in our database and 500 nondiabetic subjects, and this analysis showed the same results as that included in the manuscript (see below).

	Cases (n= 500)	Controls (n= 500)	P*
<hr/>			
Asp299Gly			
A/A	471 (94.2)	432 (86.4)	0.0001
A/G	28 (5.6)	63 (12.6)	
G/G	1 (0.2)	5 (1.0)	
<hr/>			
Thr399Ile			
C/C	466 (93.2)	436 (87.2)	0.006
C/T	33 (6.6)	59 (11.8)	

T/T

1 (0.2)

5 (1.0)

Data are presented as number of carriers (%) or proportion. * P values were computed using χ^2 tests to compare T2DM patients and nondiabetic subjects

Reviewer #3: This paper reports the findings of a case-control study and meta-analysis. This study was performed to determine whether the TLR4 Asp299Gly (rs4986790) and Thr399Ile (rs4986791) polymorphisms are associated with type 2 diabetes mellitus (T2DM). The report makes a significant contribution to the field, in general, and in particular, as it relates to the demonstration of population level genetic heterogeneity of the TLR4 Asp299Gly and Thr399Ile polymorphisms and their association with protection from type 2 diabetes mellitus. The analytic methods were of high quality and I particularly liked the meta-analysis, which was rigorously performed. This paper should be published as quickly as possible and my congratulations to the authors for an outstanding piece of scholarship. Although clearly more studies are needed, I think this type of work paves the way for molecular engineering of gene expression so as to confer protection against type 2 diabetes mellitus. I think the authors should speculate along these lines. Clearly, this paper raises the potential for thinking along those lines.

Answer: Thank you for the comments. We now included a sentence in the Discussion section about the future possibility of blocking TLR4 to protect against T2DM (page 20, last paragraph).