Universidade Federal do Rio Grande do Sul

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ESTUDO DA ASSOCIAÇÃO DE POLIMORFISMOS NO GENE RECEPTOR DO TIPO TOLL 3 (*TLR3*) E O DIABETES MELLITUS TIPO 1

Dissertação de Mestrado

Taís Silveira Assmann

Porto Alegre, outubro de 2013

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Orientadora: Profa. Dra. Daisy Crispim Moreira

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“Agir, eis a inteligência verdadeira. Serei o que quiser. Mas tenho que querer o que for. O êxito está em ter êxito, e não em ter condições de êxito. Condições de palácio têm qualquer terra larga, mas onde estará o palácio se não o fizerem ali?”

Fernando Pessoa

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"Se vi mais longe foi por estar de pé sobre ombros de gigantes" Isaac Newton

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Esta dissertação de mestrado segue o formato proposto pelo Programa de Pós-graduação em Ciências Médicas: Endocrinologia, Metabolismo e Nutrição da Faculdade de Medicina, Universidade Federal do Rio Grande do Sul, sendo apresentada na forma de um artigo de revisão e de um artigo original sobre o tema da dissertação.

- Artigo de revisão: Receptor do tipo Toll 3 (TLR3) e o desenvolvimento do Diabetes Mellitus tipo 1 (a ser submetido aos *Arquivos Brasileiros de Endocrinologia e Metabologia*).

- Artigo original: Polimorfismos no gene receptor do tipo Toll 3 *(TLR3)* estão associados com risco para o Diabetes Mellitus tipo 1 (a ser submetido ao *European Journal of Endocrinology*).

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1. Introdução

BB	*Bio-Breeding*
CV-B	Coxsackievirus B
DM	Diabetes Mellitus
DM1	Diabetes Mellitus tipo 1
IL-1	Interleucina-1
IFN-I	Interferon do tipo I
IFN-γ	Interferon-γ
IRF-3	Fator regulador de interferon-3
IRF-7	Fator regulador de interferon-7
MDA5	*Melanoma Differentiation-Associated Gene 5*
MyD88	Fator de diferenciação mielóide 88
NF-κB	Fator nuclear kappa B
PAMPs	*Pathogens-Associated Molecular Pattern*
PIC	Ácido polinosínico - policitidílico
PRRs	*Pattern recognition receptors*
RNAfd	RNA fita dupla
STAT-1	*Signal transducers and activators of transcription-1*
TLR3	Receptor do tipo Toll 3
TLRs	Receptores do tipo Toll
TRIF	*Toll-IL-1 receptor-domain-containg adaptor protein inducing INF-ß*

2. Artigo de revisão e artigo original

AH	Arterial hypertension
CVB	Coxsackievirus B
DAMPs	Damage associated molecular patterns
dsRNA	Double-stranded RNA
ECD	Ectodomain
GHb	Glycated hemoglobin
HWE	Hardy-Weinberg equilibrium
IFNs	Interferons
IL-1β	Interleucin 1β
IL-6	Interleucin 6
IRF	Interferon regulatory factor
LD	Linkage disequilibrium
MAPK	Mitogen-activated protein kinases
MDA5	Melanoma differentiation-associated gene 5
mRNA	messenger RNA
MyD88	Myeloid differentiation factor 88
NF-κB	Factor nuclear kappa B
NOD	Nucleotide-binding oligomerization domain
OR	Odds ratio
PAMPs	Pathogen-associated molecular patterns
PBMNCs	Peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
poly (I:C)	polyinosinic: polyribocytidylic acid

PRRs	Pattern-recognition receptors
RIG-I	Retinoic acid-inducible
RLHs	Retinoic acid-inducible-like helicases
SNPs	Single nucleotide polymorphisms
T1DM	Type 1 diabetes mellitus
TIR	Toll/IL-1 domain
TLR3	Toll-like receptor 3
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TRAF6	TNF receptor-associated factor 6
TRIF	TIR-domain-containing adaptor inducing IFN-β

RESUMO

Introdução: O diabetes mellitus tipo 1 (DM1) é uma doença autoimune crônica e progressiva caracterizada por descompensações metabólicas frequentemente acompanhadas por desidratação e cetoacidose. Os agentes virais parecem ter um papel importante no desencadeamento da destruição autoimune que leva ao desenvolvimento do DM1. Entre as cepas virais estudadas, a família dos enterovírus foi associada ao surgimento da doença em humanos. Um dos mediadores do dano viral é o RNA fita dupla (RNAfd) gerado durante a replicação e transcrição do RNA e DNA viral. O gene *TLR3* codifica um receptor endoplasmático pertencente à família dos *Pattern-Recognition Receptors* (PRR), o qual reconhece o RNAfd, tendo um importante papel na resposta imune inata desencadeada por infecção viral. A ligação do RNAfd ao TLR3 desencadeia a liberação de citocinas proinflamatórias, como interferons, as quais exibem uma potente ação anti-viral; assim, protegendo as células não infectadas e induzindo apoptose naquelas já contaminadas. Dessa forma, esse estudo teve como objetivo investigar a associação entre polimorfismos no gene *TLR3* e o DM1.

Métodos: As frequências dos polimorfismos rs5743313, rs11721827, rs3775291, rs13126816 e rs7668666 no gene *TLR3* foram analisadas em 476 pacientes com DM1 e em 507 indivíduos não-diabéticos saudáveis. Os haplótipos construídos a partir da combinação dos cinco polimorfismos estudados e suas frequências foram inferidos utilizando o programa Phase 2.1, o qual implementa o método estatístico bayesiano.

Resultados: Todos os genótipos estão de acordo com o esperado pelo Equilíbrio de Hardy-Weinberg. Os polimorfismos rs3775291 e rs13126816 foram associados com risco para DM1 em diferentes modelos de herança, com a associação mais forte sendo observada para o modelo aditivo [OR= 2,3 (IC 95% 1,3-4,1) e OR= 2,1 (IC 95% I 1,4-

3,2); respectivamente]. Os demais polimorfismos estudados não foram associados ao DM1. Interessantemente, a frequência de DM1 aumentou quanto maior o número de alelos mutados dos cinco polimorfismos estudados presente nos haplótipos (*p-trend*= 0,002). Além disso, em pacientes com DM1, os alelos mais raros dos polimorfismos rs5743313 e rs11721827 foram associados com menor idade de diagnóstico do DM1 e a um pior controle glicêmico.

Conclusão: Os polimorfismos rs3775291 e rs13126816 no gene *TLR3* estão associados com risco para o DM1 em indivíduos do Sul do Brasil, enquanto os polimorfismos rs5743313 e rs11721827 estão associados com idade de diagnóstico precoce e a um pior controle glicêmico. O número de alelos de risco nos haplótipos formados pelos cinco polimorfismos estudados no gene *TLR3* parece influenciar o risco para DM1, sugerindo que esses polimorfismos interagem na suscetibilidade para a doença.

ABSTRACT

Introduction: Type 1 diabetes mellitus (T1DM) is a chronic, progressive autoimmune disease characterized by metabolic decompensation often leading to dehydration and ketoacidosis. Viral agents seem to have an important role in triggering the autoimmune destruction that leads to the development of T1DM. Among several viral strains investigate so far, the enterovirus family has been consistently associated with the onset of T1DM in humans. One of the mediators of viral damage is the double-stranded RNA (dsRNA) generated during replication and transcription of viral RNA and DNA. The Toll-like receptor 3 (*TLR3*) gene codes for an endoplasmic receptor of the pattern-recognition receptors (PRRs) family that recognizes dsRNA, playing an important role in the innate immune response triggered by viral infection. Binding of dsRNA to the TLR3 triggers the release of proinflammatory cytokines, such as interferons, which exhibit potent antiviral action; thus, protecting uninfected cells and inducing apoptosis of infected ones. Therefore, this study aimed to investigate whether TLR3 polymorphisms were associated with T1DM.

Methods: Frequencies of the *TLR3* rs5743313, rs11721827, rs3775291, rs13126816 and rs7668666 polymorphisms were analyzed in 476 T1DM patients and in 507 healthy subjects. Haplotypes constructed from the combination of these polymorphisms were inferred using Bayesian statistical method.

Results: All genotypes are in agreement with those predicted by the Hardy-Weinberg equilibrium. The rs3775291 and rs13126816 polymorphisms were associated with T1DM in different inheritance models, with the strongest association being observed for the additive model [OR= 2.3 (95% CI 1.3-4.1) and OR= 2.1 (95% CI 1.4-3.2); respectively]. The other three polymorphisms were not significantly associated with

T1DM. Interestingly, the prevalence of T1DM was higher as more risk alleles of the five polymorphisms were present (P trend = 0.002). Moreover, in T1DM patients, the minor alleles of the rs5743313 and rs117221827 polymorphisms were associated with an early age at diagnosis and worse glycemic control.

Conclusion: The *TLR3* rs3775291 and rs13126816 polymorphisms are associated with risk for T1DM in Southern Brazilian subjects, while the rs5743313 and rs11721827 polymorphisms are associated with age at T1DM diagnosis and worst glycemic control. The number of risk alleles of the five *TLR3* polymorphisms in the haplotypes seems to influence the risk for T1DM, suggesting that these polymorphisms might interact in the susceptibility for the disease.

INTRODUÇÃO

De acordo com a Federação Internacional de Diabetes (IDF) (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 providências se fazem necessárias para modificar a trajetória da doença, caso isso não ocorra, 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 5% do total de casos apresenta DM tipo 1 (DM1). Estudos epidemiológicos demonstram que a taxa de incidência do DM1 está crescendo nas últimas décadas em todo o mundo, particularmente em crianças, com estimativa de 70.000 novos casos por ano (2, 3). Isso é preocupante, pois o DM1 é a doença crônica grave mais comum na infância, uma vez que o número de células-beta pancreáticas é drasticamente diminuído, chegando a zero ou próximo disto e deixando o paciente dependente de insulina exógena para a sobrevivência (4).

O DM1 é uma doença autoimune de etiologia múltipla, onde há a associação complexa de fatores genéticos, ambientais e imunológicos. Em indivíduos geneticamente predispostos, a doença parece ser desencadeada por fatores ambientais, tais como, infecções virais, fatores perinatais, proteínas do leite, componentes dos cereais, nitratos e imunizações, bem como a "hipótese da higiene" que postula que a menor exposição a patógenos contribui para a autoimunidade (5, 6).

Infecções por vírus estão entres os principais fatores ambientais relacionados ao DM1. Até o momento, treze diferentes vírus foram associados ao desenvolvimento da doença em humanos e em vários modelos animais, incluindo os enterovírus, como Coxsackievirus B (CV-B), rotavírus, vírus da caxumba e citomegalovírus (7, 8).

A mais bem documentada correlação entre um vírus e o desencadeamento do DM1 é para os enterovírus (9). O CV-B, um tipo de enterovírus, foi previamente isolado de um paciente que faleceu logo após o diagnóstico de DM1 devido a complicações agudas (10). Recentemente, a infecção das células-beta pancreáticas por CV-B4 foi identificada em três de seis pacientes com DM1 de início recente (11). Uma extensa série de amostras pancreáticas obtidas de pacientes com DM1 também demonstrou positividade para o capsídio viral VP1 de CV-B em 61% dos pacientes e em apenas 6% dos controles (11).

Os mecanismos de lesão imunológica da célula-beta mediada por vírus não foram totalmente elucidados, mas parecem se relacionar à lesão viral direta associada ao dano mediado por citocinas produzidas por células do sistema imune e pela própria célula-beta (7, 12, 13). As células-beta pancreáticas são particularmente suscetíveis ao efeito de citocinas inflamatórias, que são secretadas durante a resposta imune, gerando o processo de insulite. Assim, o DM1 pode ser visto como uma falha nas vias intracelulares de defesa imune das células-beta, levando-as ao processo de apoptose ao invés de promover sua proliferação e recuperação após o dano autoimune (14).

Um dos mediadores do dano viral é o RNA fita dupla (RNAfd), um subproduto gerado durante a replicação viral. O RNAfd acumula-se nas células infectadas e desencadeia uma série de atividades antivirais por parte da célula hospedeira, como a produção de interferon do tipo I (IFN-I), quimiocinas, ativação da proteína cinase dependente de RNA fita simples (PKR) e a inibição da síntese protéica (15). Essas respostas virais podem ser mimetizadas pelo RNAfd sintético, o ácido polinosínico-policitidílico (PIC) (16, 17). *In vivo*, PIC desencadeia hiperglicemia em linhagens de ratos BB (*Bio-Breeding*) resistentes ao DM1 e acelera o desenvolvimento dessa doença em uma linhagem de ratos BB predispostos ao DM1 (16). *In vitro*, PIC inibe a produção

de insulina estimulada por glicose em ilhotas de camundongos e, quando usado em combinação com interferon-γ (INF-γ), reduz a função e diminui a viabilidade das ilhotas pancreáticas (18, 19).

A imunidade inata representa a primeira linha de defesa de humanos e outros organismos pluricelulares contra microorganismos patogênicos (20). 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) (21, 22). Estudos recentes identificaram três grupos principais de PRRs: *Toll-like receptors* (TLRs), *retinoic acid-inducible gene I* (RIG-I)-*like helicases* (RLHs) e *nucleotide-oligomerization domain* (NOD)-*like receptors* (NLRs) (23).

Durante a infecção viral, PAMPs específicos (principalmente RNA de fita simples/dupla) são reconhecidos 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 IFN-I. 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 (21, 23).

Entre os PRRs, os mais extensivamente estudados são os TLRs, que são glicoproteínas transmembranas originalmente identificadas em *Drosophila* (22, 24). Até o momento, onze membros da família TLR foram identificados em mamíferos (25, 26). 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 e fungos. TLR3, TLR7, TLR8 e

TLR9 são localizados em endossomos e seus ligantes, principalmente ácidos nucléicos virais ou bacterianos, requerem internalização nos endossomos (26-29).

Diversos membros da família dos TLRs têm surgido como sensores-chaves da imunidade inata para o reconhecimento de componentes virais, tais como ácidos nucléicos e glicoproteínas do envelope. A ligação do patógeno aos TLRs desencadeia a produção de IFN-1, citocinas proinflamatórias 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 (30).

Entre os TLRs humanos, os receptores tipo 1, 2, 3 e 7 são ativados perante a exposição antigênica viral. Sendo assim, cada um dos genes que codificam esses receptores são candidatos para o estudo da interação do sistema imune inato com partículas virais e a ativação do sistema imune adaptativo na patogênese do DM1. O TLR3 é o receptor desta família que responde especificamente ao RNAfd derivado do metabolismo viral (30).

A maioria dos TLRs compartilha componentes sinalizadores com o receptor da interleucina 1 (IL-1), incluindo a cinase associada ao receptor da IL-1 e as moléculas adaptadoras citoplasmáticas MyD88 e TRAF6, sendo o TLR/My88 descrito em camundongos como importante para o reconhecimento de patógenos (25, 31). Porém, o TLR3 utiliza uma via independente de MyD88, envolvendo o *Toll-IL1 receptor-domain-containg adaptor protein inducing INF-β* (TRIF) (25). A ativação do TRIF induzida pelo RNAfd leva à indução dos fatores de transcrição NF-κB e do IRF (fator regulador de interferon)-3 (32). Após a ativação mediada pelo TLR3, a forma fosforilada do IRF-3 desloca-se para o núcleo e age em conjunto com o NF-κB para induzir um aumento na expressão de IFN-I, o qual, após ser secretado, irá ativar a

expressão de quimiocinas e citocinas proinflamatórias através da via STAT1/2 (32-34). O IRF-7 também é ativado e está envolvido na produção tardia de IFN α/β, desta forma amplificando a sinalização do IFN-I (35).

O NF-κB é um regulador-chave da resposta imune e a indução de genes dependentes de NF-κB é crucial para o desenvolvimento de uma resposta inflamatória adequada. Estudos realizados em células-beta demonstraram que a ativação do NF-κB, diferente da maioria dos outros tipos celulares, tem, principalmente, efeitos proapoptóticos (36-38) e que a sua inibição previne a morte das células-beta (39). Além disso, o bloqueio da cascata sinalizadora do STAT-1 também parece proteger as células-beta dos efeitos apoptóticos da associação RNAfd externo + INF-γ (40). Desta forma, RNAfd externo + INF-γ induzem a apoptose das células-beta por duas vias complementares, via ativação do NF-κB, dependente do RNAfd, e do STAT-1, dependente do INF-γ.

Além disso, deve-se ser enfatizado que, em alguns casos, moléculas endógenas também podem iniciar uma resposta imunológica via TLRs e, como consequência, gerar o processo de autoimunidade (41). Dados recentes de experimentos com modelos animais com DM1 corroboram a ideia de que a super-regulação dos TLRs pode, em determinadas situações, levar à resposta pró-inflamatória e à destruição das células-beta. A grande questão é se existem vias de sinalização anormal em indivíduos com predisposição genética, gerando uma alteração na capacidade do sistema imune inato em responder aos patógenos e se estas aberrações potenciais estão envolvidas nos mecanismos iniciais que levam ao DM1 (42).

No DM1 autoimune, os receptores de RNAfd podem ser inadequadamente estimulados por ácidos nucléicos endógenos gerados pela apoptose ou necrose celular ou ainda por infecções virais que não têm como alvo as células-beta. Tais eventos

podem levar a uma excessiva produção de IFN-I, que ativa os linfócitos T autorreativos latentes. Em contraste, durante a infecção viral com tropismo pelas células-beta, há a atividade dos sensores de RNAfd, particularmente TLR3 em células hematopoiéticas, o que pode gerar uma resposta exacerbada de IFN-I, bem como o descontrole da replicação viral e desencadeamento do DM1. Interessantemente, a análise do tecido pancreático de pacientes com DM1 de diagnóstico precoce demonstra diferentes padrões histológicos evidenciando tanto lesões autoimunes quanto virais (11). Enquanto a análise de polimorfismos com hiperfunção ou sem alteração da função do *MDA5* (*Melanoma Differentiation-Associated Gene 5* ou *IFIH1*) de humanos predispõem ao DM1 autoimune, polimorfismos de diminuição de função no *TLR3* (e possivelmente no *MDA5*) podem predispor a suscetibilidade ao DM1 induzido por vírus presentes no ambiente (43, 44).

Até o momento, apenas o estudo de Pirie *et al*. (45), em indivíduos sul-africanos, mostrou a associação direta entre polimorfismos no gene *TLR3* e risco para o desenvolvimento de DM1. Assim, mais estudos são necessários para investigar a associação entre polimorfismos no gene *TLR3* e o DM1 em diferentes populações. Nesse contexto, o objetivo do presente estudo foi investigar a associação entre os polimorfismos rs5743313 (C/T), rs3775291 (A/G), rs11721827 (A/C), rs13126816 (A/G) e rs7668666 (A/C) no gene *TLR3* e DM1 em uma população branca do Sul do Brasil.

Bibliografia

1. International Diabetes Federation. Epidemiology [cited 25 de setembro de 2013]. Available from: **www.idf.org**.
2. Onkamo P, Väänänen S, Karvonen M, Tuomilehto J. Worldwide increase in incidence of Type I diabetes--the analysis of the data on published incidence trends. Diabetologia. 1999;42(12):1395-403.
3. Knip M. Descriptive epidemiology of type 1 diabetes--is it still in? Diabetologia. 2012;55(5):1227-30.
4. American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2013;36 Suppl 1:S67-74.
5. Knip M, Veijola R, Virtanen SM, Hyöty H, Vaarala O, Akerblom HK. Environmental triggers and determinants of type 1 diabetes. Diabetes. 2005;54 Suppl 2:S125-36.
6. Beyan H, Wen L, Leslie RD. Guts, germs, and meals: the origin of type 1 diabetes. Curr Diab Rep. 2012;12(5):456-62.
7. Jun HS, Yoon JW. The role of viruses in type I diabetes: two distinct cellular and molecular pathogenic mechanisms of virus-induced diabetes in animals. Diabetologia. 2001;44(3):271-85.
8. Filippi CM, von Herrath MG. Viral trigger for type 1 diabetes: pros and cons. Diabetes. 2008;57(11):2863-71.
9. Coppieters KT, Boettler T, von Herrath M. Virus infections in type 1 diabetes. Cold Spring Harb Perspect Med. 2012;2(1):a007682.
10. Yoon JW, Austin M, Onodera T, Notkins AL. Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. N Engl J Med. 1979;300(21):1173-9.
11. Dotta F, Censini S, van Halteren AG, Marselli L, Masini M, Dionisi S, et al. Coxsackie B4 virus infection of beta cells and natural killer cell insulitis in recent-onset type 1 diabetic patients. Proc Natl Acad Sci U S A. 2007;104(12):5115-20.
12. Eizirik DL, Darville MI. beta-cell apoptosis and defense mechanisms: lessons from type 1 diabetes. Diabetes. 2001;50 Suppl 1:S64-9.
13. Yoon JW, Jun HS. Viruses cause type 1 diabetes in animals. Ann N Y Acad Sci. 2006;1079:138-46.
14. Gysemans CA, Ladrière L, Callewaert H, Rasschaert J, Flamez D, Levy DE, et al. Disruption of the gamma-interferon signaling pathway at the level of signal transducer and activator of transcription-1 prevents immune destruction of beta-cells. Diabetes. 2005;54(8):2396-403.
15. Jacobs BL, Langland JO. When two strands are better than one: the mediators and modulators of the cellular responses to double-stranded RNA. Virology. 1996;219(2):339-49.
16. Sobel DO, Newsome J, Ewel CH, Bellanti JA, Abbassi V, Creswell K, et al. Poly I:C induces development of diabetes mellitus in BB rat. Diabetes. 1992;41(4):515-20.
17. Der SD, Yang YL, Weissmann C, Williams BR. A double-stranded RNA-activated protein kinase-dependent pathway mediating stress-induced apoptosis. Proc Natl Acad Sci U S A. 1997;94(7):3279-83.
18. Heitmeier MR, Scarim AL, Corbett JA. Double-stranded RNA inhibits beta-cell function and induces islet damage by stimulating beta-cell production of nitric oxide. J Biol Chem. 1999;274(18):12531-6.

19. Liu D, Darville M, Eizirik DL. Double-stranded ribonucleic acid (RNA) induces beta-cell Fas messenger RNA expression and increases cytokine-induced beta-cell apoptosis. Endocrinology. 2001;142(6):2593-9.
20. Medzhitov R. Recognition of microorganisms and activation of the immune response. Nature. 2007;449(7164):819-26.
21. Kumar H, Kawai T, Akira S. Pathogen recognition in the innate immune response. Biochem J. 2009;420(1):1-16.
22. Meylan E, Tschopp J, Karin M. Intracellular pattern recognition receptors in the host response. Nature. 2006;442(7098):39-44.
23. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010;140(6):805-20.
24. Medzhitov R, Preston-Hurlburt P, Janeway CA. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature. 1997;388(6640):394-7.
25. Takeda K. [Toll-like receptor]. Nihon Rinsho Meneki Gakkai Kaishi. 2005;28(5):309-17.
26. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity. 2011;34(5):637-50.
27. Takeda K, Akira S. Toll-like receptors in innate immunity. Int Immunol. 2005;17(1):1-14.
28. Amarante MK, Watanabe MA. Toll-like receptor 3: involvement with exogenous and endogenous RNA. Int Rev Immunol. 2010;29(6):557-73.
29. Moresco EM, LaVine D, Beutler B. Toll-like receptors. Curr Biol. 2011;21(13):R488-93.
30. Xagorari A, Chlichlia K. Toll-like receptors and viruses: induction of innate antiviral immune responses. Open Microbiol J. 2008;2:49-59.
31. Egan CE, Sukhumavasi W, Butcher BA, Denkers EY. Functional aspects of Toll-like receptor/MyD88 signalling during protozoan infection: focus on Toxoplasma gondii. Clin Exp Immunol. 2009;156(1):17-24.
32. Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K, et al. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. J Immunol. 2002;169(12):6668-72.
33. Sato S, Sugiyama M, Yamamoto M, Watanabe Y, Kawai T, Takeda K, et al. Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. J Immunol. 2003;171(8):4304-10.
34. Taniguchi T, Takaoka A. The interferon-alpha/beta system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors. Curr Opin Immunol. 2002;14(1):111-6.
35. Sato M, Suemori H, Hata N, Asagiri M, Ogasawara K, Nakao K, et al. Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. Immunity. 2000;13(4):539-48.
36. Giannoukakis N, Mi Z, Rudert WA, Gambotto A, Trucco M, Robbins P. Prevention of beta cell dysfunction and apoptosis activation in human islets by adenoviral gene transfer of the insulin-like growth factor I. Gene Ther. 2000;7(23):2015-22.
37. Cardozo AK, Heimberg H, Heremans Y, Leeman R, Kutlu B, Kruhøffer M, et al. A comprehensive analysis of cytokine-induced and nuclear factor-kappa B-

dependent genes in primary rat pancreatic beta-cells. J Biol Chem. 2001;276(52):48879-86.
38. Heimberg H, Heremans Y, Jobin C, Leemans R, Cardozo AK, Darville M, et al. Inhibition of cytokine-induced NF-kappaB activation by adenovirus-mediated expression of a NF-kappaB super-repressor prevents beta-cell apoptosis. Diabetes. 2001;50(10):2219-24.
39. Liu D, Cardozo AK, Darville MI, Eizirik DL. Double-stranded RNA cooperates with interferon-g and IL-1b to induce both chemokine expression and nuclear factor-kB-dependent apoptosis in pancreatic beta-cells: potential mechanisms for viral-induced insulitis and beta-cell death in type 1 diabetes mellitus. Endocrinology. 2002;143(4):1225-34.
40. Rasschaert J, Ladrière L, Urbain M, Dogusan Z, Katabua B, Sato S, et al. Toll-like receptor 3 and STAT-1 contribute to double-stranded RNA+ interferon-gamma-induced apoptosis in primary pancreatic beta-cells. J Biol Chem. 2005;280(40):33984-91.
41. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science. 2004;303(5663):1526-9.
42. Lien E, Zipris D. The role of Toll-like receptor pathways in the mechanism of type 1 diabetes. Curr Mol Med. 2009;9(1):52-68.
43. Casanova JL, Abel L, Quintana-Murci L. Human TLRs and IL-1Rs in host defense: natural insights from evolutionary, epidemiological, and clinical genetics. Annu Rev Immunol. 2011;29:447-91.
44. McCartney SA, Vermi W, Lonardi S, Rossini C, Otero K, Calderon B, et al. RNA sensor-induced type I IFN prevents diabetes caused by a β cell-tropic virus in mice. J Clin Invest. 2011;121(4):1497-507.
45. Pirie FJ, Pegoraro R, Motala AA, Rauff S, Rom L, Govender T, et al. Toll-like receptor 3 gene polymorphisms in South African Blacks with type 1 diabetes. Tissue Antigens. 2005;66(2):125-30.

ARTIGO DE REVISÃO

RECEPTOR DO TIPO TOLL 3 (TLR3) E O DESENVOLVIMENTO DO DIABETES MELLITUS TIPO 1

TOLL-LIKE RECEPTOR (TLR3) AND THE DEVELOPMENT OF TYPE 1 DIABETES MELLITUS

RECEPTOR DO TIPO TOLL 3 (TLR3) E O DESENVOLVIMENTO DO DIABETES MELLITUS TIPO 1

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Short title: TLR3 and type 1 diabetes.

SUMMARY

Type 1 diabetes mellitus (T1DM) is a chronic, progressive autoimmune disease characterized by metabolic decompensation often leading to dehydration and ketoacidosis. Viral agents seem to have an important role in triggering the autoimmune destruction that leads to the development of T1DM. Among several viral strains investigate so far, the enterovirus family has been consistently associated with the onset of T1DM in humans. One of the mediators of viral damage is the double-stranded RNA (dsRNA) generated during replication and transcription of viral RNA and DNA. The Toll-like receptor 3 (*TLR3*) gene codes for an endoplasmic receptor of the pattern-recognition receptors (PRRs) family that recognizes dsRNA, playing an important role in the innate immune response triggered by viral infection. Binding of dsRNA to the TLR3 triggers the release of proinflammatory cytokines, such as interferons, which exhibit potent antiviral action; thus, protecting uninfected cells and inducing apoptosis of infected ones. Therefore, *TLR3* gene is a good candidate for the development of T1DM. Within this context, the objective of the present review was to address the role of *TLR3* gene in the development of T1DM.

Keywords

Autoimmunity; type 1 diabetes mellitus; viral infection; Toll-like receptor 3 (TLR3).

SUMÁRIO

O diabetes mellitus tipo 1 (DM1) é uma doença autoimune crônica e progressiva caracterizada por descompensações metabólicas frequentemente acompanhadas por desidratação e cetoacidose. Os agentes virais parecem ter um papel importante no desencadeamento da destruição autoimune que leva ao desenvolvimento do DM1. Entre as cepas virais estudadas, a família dos enterovírus foi associada ao surgimento da doença em humanos. Um dos mediadores do dano viral é o RNA fita dupla (RNAfd) gerado durante a replicação e transcrição do RNA e DNA viral. O gene *TLR3* codifica um receptor endoplasmático pertencente à família dos *Pattern-Recognition Receptors* (PRR), o qual reconhece o RNAfd, tendo um importante papel na resposta imune inata desencadeada por infecção viral. A ligação do RNAfd ao TLR3 desencadeia a liberação de citocinas proinflamatórias, como interferons, as quais exibem uma potente ação anti-viral; desta forma, protegendo as células não-infectadas e induzindo apoptose naquelas já contaminadas. Portanto, o gene *TLR3* é um bom candidato ao desenvolvimento do DM1. Dentro deste contexto, o objetivo da presente revisão foi abordar o papel do gene *TLR3* no desenvolvimento do DM1.

Descritores

Autoimunidade; diabetes mellitus tipo 1; infecção viral; Toll-like receptor 3 **(**TLR3)

INTRODUCTION

Type 1 diabetes mellitus (T1DM), which accounts for 5-10% of all cases of diabetes, is characterized by severe autoimmune destruction of insulin-producing beta-cells in the pancreas by T lymphocytes and macrophages infiltrating the islets of Langerhans (1, 2). Consequently, subjects with T1DM are usually dependent on insulin injections for life (3).

Inflammation of the islets (insulitis) probably develops within a context of a "dialog" between immune cells and pancreatic beta-cells. This dialog is mediated by cytokines and chemokines, which are released by immune cells and beta-cells, as well as by other immunogenic signals delivered by dying beta-cells. This may lead to induction and amplification of the inflammatory process, but in some cases, may lead to the resolution of insulitis (4). Furthermore, the course of beta-cell inflammation and its potential progression to clinical T1DM depends on a complex interaction between a strong genetic component and a diversity of environmental triggers (5, 6).

Among the several loci associated with T1DM, the human leukocyte antigen (*HLA*) class II locus is undoubtedly the leading genetic risk factor for T1DM, accounting for 30–50% of the genetic risk for this disease (7). Other genes are associated with minor effects on T1DM risk when compared with HLA, such as the *insulin* gene, the cytotoxic T-lymphocyte associated protein 4 (*CTLA4*) gene, the protein tyrosine phosphatase, nonreceptor type 22 (*PTPN22*) gene, and other genes discovered through genome-wide association studies (GWAS) (7, 8).

Several epidemiologic studies have found that environmental factors, such as viral pathogens operating early in life, seem to trigger the autoimmune destruction of beta-cells in genetically susceptible subjects (9-11). Host defense against invading

microbial microorganisms is elicited by the immune system, which consists of two components: innate immunity and adaptive immunity. Both components of immunity recognize invading microorganisms as non-self; thus, triggering immune responses to eliminate them (12).

Innate immunity is the first line of defense against viruses, bacteria and fungi. Detection of viruses or others invading microorganisms is carried out by a wide range of cell receptors of the pattern-recognition receptors (PRRs) family, which recognize highly conserved pathogen-associated molecular patterns (PAMPs), such as the double-stranded RNA (dsRNA) generated during the life cycle of most viruses (13, 14). Innate immune cells, such as macrophages and dendritic cells, kill invading microorganisms through phagocytosis or production of cytokines, chemokines and type I interferons. These interferons (IFNs) have potent antiviral actions, protecting uninfected cells and inducing apoptosis of those already infected (15). Moreover, activation of innate immunity is an important step to the development of antigen-specific adaptive immune system, consisting of B lymphocytes, which produce specific antibodies against the invading pathogen, and T lymphocytes, which secrete cytokines that will induce elimination of infected cells by exerting cytotoxic effects or by signaling B lymphocytes (12, 14).

Many types of PRRs have been identified so far, including toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs) (15, 16). Of these, the most extensively studied are TLRs, which are type I transmembrane glycoproteins originally identified in *Drosophila* (13, 17). Human homologs are known to comprise at least 10 members with different response triggers (18). Amongst the human TLRs, TLR3 has been shown to respond to dsRNA, a replication product of some virus, and it is

expressed at high levels in human and mouse pancreatic beta-cells and in antigen-presenting dendritic cells (4, 19). Therefore, *TLR3* is a plausible candidate gene for T1DM susceptibility. Within this context, the objective of the present review was to address the role of *TLR3* gene in the development of T1DM.

THE VIRUS INFECTION HYPOTHESIS AS A TRIGGER FOR THE DEVELOPMENT FOR T1DM

The hypothesis that viral infections are involved in T1DM is based on epidemiological studies and anecdotal data (11, 20, 21). One of the major observations that support a role for viruses in T1DM is that concordance rates for T1DM in monozygotic twins are only ~50% instead of the expected 100% if the characteristic would be explained only by genetic factors. Also, there was an increase in the T1DM incidence over the past 50 years in many countries, which cannot be explained by alterations in genetic risk factors. In addition, migration studies show that the incidence of T1DM in offspring of subjects who had moved from a low-incidence to a high-incidence area is increased compared to the original incidence observed in the area of origin. Importantly, viruses and virus-specific antibodies can be detected in newly diagnosed patients with T1DM (10, 20, 22). Indeed, a number of viruses have been associated with T1DM in humans, such as enteroviruses, rubella virus, mumps virus, rotaviruses, parvoviruses, and cytomegalovirus (20, 23). Overall, this data demonstrate a correlation between particular virus infections and human T1DM; however, this correlation neither establishes a direct link between microbial infections and the disease nor provides mechanistic insights into the autoimmune process leading to disease (20).

The most well documented correlation between a virus and T1DM has been for enteroviruses, non-enveloped single-stranded RNA viruses belonging to the picornavirus family (10, 21). Enterovirus infections are more frequent in siblings developing T1DM compared to nondiabetic siblings, and enterovirus antibodies are elevated in pregnant women whose children later develop T1DM (24). Interestingly, epidemiological studies of the seasonality of development of anti-beta-cell antibodies in a group of subjects at increased risk of T1DM showed an increased incidence of autoantibodies during winter, which correlated with a period of increased enteroviral infection rates (11, 23). Recently, Oikarinen *et al*. (25) isolated enteroviruses from intestinal biopsies in 75% of T1DM patients and in 10% of nondiabetic subjects, indicating that a substantial proportion of T1DM patients have an ongoing enterovirus infection in gut mucosa. Elshebani *et al*. (26) found that enteroviruses isolated from newly diagnosed T1DM patients could infect human islets *in vitro*, reducing their ability to secrete insulin in response to high glucose, and increasing the number of dead cells. In addition, a recent meta-analysis, including 24 papers and 2 abstracts, showed a significant association between enterovirus infection and T1DM-related autoimmunity (OR = 3.7, 95% CI = 2.1-6.8) or clinical T1DM (OR = 9.8, 95% CI = 5.5 – 17.4) (27).

Among the enteroviruses, the most significant association with T1DM was with Coxsackievirus (CVB), which exhibit specific tropism for the pancreas (28-30). Serological and PCR analyses have shown that CVB are expressed more frequently in T1DM patients than in healthy subjects [reviewed in (20)]. One CVB4 strain was isolated from the pancreas of a child who died from diabetic ketoacidosis, and this virus caused diabetes when inoculated in a susceptible mouse strain (31). Furthermore, Dotta *et al*. (32) identified CVB4 in 50% of samples collected from patients with T1DM, and

these enteroviruses were able to infect human islets *in vitro*, impairing insulin secretion in response to glucose.

Based on the studies presented above, enteroviruses appear to be associated with a fraction of T1DM cases. Nevertheless, if enteroviruses play a major role in the T1DM pathogenesis, how could we explain the increase in T1DM incidence in countries where exposure to these microorganisms has been dropping, such as Finland? Are the data showing that T1DM can be caused by viral infections compatible with the hygiene hypothesis? [see reference (33) for more details]. Interestingly, data in NOD mice revealed that CVB, and the B4 strain in particular, infect the exocrine portion of the pancreas and provoke diabetes in animals with established insulitis (34). Interestingly, when administered earlier in the life, CVB inoculation is associated with a strong protection for diabetes, implying that the timing of the infection plays a key role in disease development (35). Taking into account the studies in NOD mice, Coppieters *et al*. (10) suggested that the lack of exposure to enteroviruses in developed countries results in a reduced frequency of subjects with protective immunity through early childhood infections. When islet inflammation occurs in these subjects, they would be more susceptible to an enteroviral infection that has the potential to initiate autoreactivity and beta-cell damage.

Animal models have provided important data about the possible mechanisms of enterovirus-induced beta-cell damage. They have shown that several mechanisms may be involved and either a direct infection of beta-cells, molecular mimicry or bystander activation of autoreactive immune clones induce beta-cell damage [see reference (21) for more details]. Studies on human pancreata or cultured islets have shown that there are considerable variations in the adverse effects of enteroviruses on beta-cells, not only between various viral serotypes, but also between strains of the same serotype (23).

Viral infections might be able of "unmasking" beta-cells for recognition by $CD8^+$ T-cells by promoting IFN production and upregulation of MHC class I molecules on beta-cells. These events may be sufficient to mark pancreatic islets for autoimmune attack (23). However, the exact mechanisms how viruses cause T1DM in humans remain to be determined.

TOLL-LIKE RECEPTORS (TLRs)

Among the most important families of PRRs are the TLRs which recognize, with selectivity, a large number of PAMPs derived from microbes (17). Mammalian TLRs also respond to host-derived molecules that are released from injured tissues and cells, termed damage-associated molecular patterns (DAMPs) (36). TLR-ligand binding plays a key role in innate immunity and subsequent acquired immunity against microbial infection or tissue injury (37).

Structurally, TLRs are type I transmembrane-signaling PRRs. Their extracellular domain includes a repetitive structure rich in leucine residues, called leucine-rich repeat (LRR), and which is involved in ligand recognition. A transmembrane domain determines their cellular localization, and the intracellular portion contains a similar structure to that of IL-1 receptor, known as Toll/IL-1 (TIR) domain, which is essential for triggering downstream signaling pathways (38-40). So far, eleven TLRs have been identified in humans and 13 in mice, recognizing different PAMPs and DAMPs (14, 15, 39). **Table 1** shows the 11 human TLRs with their specific ligands. The ligands for TLR8 and TLR10 remain unknown (41). The human *TLR11* gene has a stop codon within an open reading frame and thus appears to be non-functional; the above findings provide many insights into host-microbe interactions through innate immune receptors (42).

TLRs are located either on the cell surface or in intracellular compartments. Cell-surface TLRs, including TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10, are essential in recognizing bacterial cell wall components, bacterial flagellin, viral particles, and other unidentified pathogenic components. TLR3, TLR7, TLR8, and TLR9 are localized in endosomes, and their ligands, mainly bacterial or viral nucleic acids, require internalization to the endosome before signaling is possible (**Table 1**) (12, 37, 41, 43).

TLRs are widely expressed in various immune cells, including dendritic cells, macrophages, B cells, specific types of T cells, and even on non-immune cells, such as epithelial cells, endothelial cells, and fibroblasts (44, 45). Expression of TLRs is not static but rather is regulated rapidly in response to pathogens, a variety of cytokines, and environmental stresses (45). Interestingly, expression of TLRs at non-immune cells appears to have an important role in the first line of defense against microbial invasions at these sites (38).

After ligand binding, TLR signaling proceeds via two pathways: the myeloid differentiation factor 88 (MyD88)-mediated pathway, and the TIR-domain-containing adaptor inducing IFN-β (TRIF)-mediated pathway. The former pathway causes the activation of the transcription factor NF-κB and various mitogen-activated protein kinases (MAPK), which will activate several genes contributing to inflammatory reactions. The latter pathway causes induction of IFNs, whose stimulation leads cells to enter in an antiviral state. TLR3 only activates the TRIF-mediated pathway, leading to the activation of interferon regulatory factor (IRF)-3, an important transcription factor which induces the expression of type 1 IFNs and the indirect upregulation of IFN-dependent genes such as IFN-inducible protein 10 (IP-10) and inducible nitric oxide synthase (iNOS). Moreover, TLR3 also activates NF-κB, which upregulates the

production of proinflammatory cytokines such as IL-1β, IL-6 and TNF. TLR4 stimulates both pathways, activating NF-κB and inducing type 1 IFNs production. All other TLRs activate exclusively the MyD88-dependent pathway (15, 46, 47).

To sum up, the main functions of TLRs are induction of an inflammatory response and establishment of adaptive immunity. TLRs activate tissue-resident macrophages to produce proinflammatory cytokines, including TNF, IL-1β and IL-6, which coordinate local and systemic inflammatory responses. These cytokines then activate surrounding cells to produce chemokines or adhesion molecules, thereby recruiting several inflammatory cells to the inflammation site. Recruited macrophages or neutrophils are activated and ingest invading pathogens through internalizing PRRs. Afterward, those cells kill the pathogens by producing nitric oxide, reactive oxygen species or defensins (39, 48).

Several studies performed in animal models of T1DM have implicated TLR pathways in mechanisms linked with both diabetes induction and prevention. In both the NOD mouse and diabetes-prone BB rat, TLR upregulation can suppress diabetes. In the BioBreeding Diabetes Resistant rat; however, diabetes induced by virus infection involves the upregulation of TLR9 pathways, and generic TLR upregulation synergizes with virus infection on diabetes induction. Studies performed in mouse models of T1DM with spontaneous or induced diabetes implicate TLR1, TLR2, TLR3, and TLR7 in disease mechanisms [reviewed in (22)]. However, only a few studies analyzed the associations between polymorphisms in the *TLR* genes and susceptibility for T1DM. For example, Bjørnvold *et al*. (49) reported that both T1DM and allergic asthma were significantly associated with the rs3804100 T allele in the *TLR2* gene, and further associated with the haplotype including this polymorphism, possibly representing a susceptibility locus common for the two diseases. Park *et al*. (50) reported that the C/C

genotype of the 1350 polymorphism in the *TLR2* gene was associated with protection for T1DM. Moreover, the homozygous haplotype constituted by the T allele of the 597 polymorphism and the C allele of the 1350 polymorphism was associated with strong protection for T1DM. Cooper *et al*. (51) analyzed a total of 1715 SNPs from the Wellcome Trust Case Control Consortium GWAS study of T1DM, and found an association between T1DM and a SNP in chromosome Xp22 (rs5979785), located 30 kb centromeric of the *TLR7* and *TLR8* genes. The authors suggested it is possible that this SNP, or variants in linkage disequilibrium with it, could alter *TLR7* and/or *TLR8* expression and, therefore, modify the risk for T1DM.

Toll-like Receptor 3 (TLR3)

Viral dsRNAs are potent inducers of type 1 IFN (IFN-α and IFN-β) antiviral responses and were reported to initiate its signaling *via* TLR3 (22). In addition to TLR3, dsRNA is recognized by the RLHs, RIG-I and melanoma differentiation-associated gene 5 (MDA5 or IFIH1). TLR3 and RIG-I/MDA5 differ in their cellular location and ligand specificities, and induce antiviral responses via different signaling pathways (37).

TLR3 is a type I transmembrane receptor with a molecular mass of 125 kDa that has several distinct structural domains, as already mentioned (52). Dimerization of TLR3 is required for ligand binding, and dsRNA molecules must be at least 40 to 50 bp in length in order to induce TLR3 signaling (52). TLR3 was originally identified to recognize a synthetic analog of dsRNA, the polyinosinic:polyribocytidylic acid [poly(I:C)], which was found to be the most potent IFN inducer (37). TLR3 also recognizes genomic RNA from dsRNA viruses (including reoviruses) and dsRNA produced during replication of single-stranded RNA viruses (such as influenza A virus,

encephalomyocarditis virus, and West Nile virus) and double-stranded DNA viruses (herpes simplex virus and murine cytomegalovirus) (43, 53). TLR3 can sense viral dsRNA in both the extracellular compartment as well as in endosomes. However, spatial localization seems to be important for recognition of viral dsRNA by TLR3, since cell-associated dsRNA has been found to be a more potent activator of TLR3 than soluble dsRNA (52).

The *TLR3* gene is located on chromosome 4q35 (**Figure 1**), and comprises five exons with a 15942 bp transcript encoding a 3057 bp mRNA and a 904 residue protein (54). *TLR3* mRNA has been detected in a number of human tissues including placenta, pancreas, lung, liver, heart, lymph nodes, and brain. Furthermore, *TLR3* transcripts have been found in a variety of human and mouse immune cells, including T lymphocytes, natural killer cells, macrophages, mast cells and $\gamma\delta$ T cells. Human fibroblasts and epithelial cells express TLR3 both intracellularly and on the cell surface while monocyte-derived immature dendritic cells and myeloid dendritic cells only express TLR3 intracellularly, which demonstrates that cells utilize different strategies to sense viral invasion and initiate antiviral responses (37, 52).

In contrast to other TLR ligands, dsRNA signaling occurs via MyD88-independent pathways. For example, dsRNA binds TLR3 with the resultant recruitment of the adaptor molecule TRIF via a TIR-TIR domain interaction (**Figure 2**). Following the activation of endosomal TLR3 by dsRNA, TRIF co-localizes with TLR3 and dimerizes through its TIR domain and C-terminal region. This induces a conformational change that allows downstream signaling molecules access to their binding sites (52, 55). Then, TRIF recruits TNF receptor-associated factor 6 (TRAF6), forming a complex containing TGF-β-activated kinase-1 (TAK1), TAK-1 binding protein 2 (TAB2), and protein kinase R, which mediates downstream NF-κB activation. Protein kinase R also

contributes to the dsRNA-induced activation of the p38 MAPK pathway by interaction with MAP kinase kinase 6 (MKK6). TRIF also recruits TRAF3, TRAF-family-member-associated NF-κB activator binding kinase 1 (TBK1) and inducible IκB kinase (IKKi), which phosphorylate IRF-3 and IRF-7 factors. After phosphorylation, activation and dimerization, IRF-3 and IRF-7 translocate into the nucleus and induce the expression of type 1 IFNs, which, after being secreted, will activate the expression of proinflammatory cytokines and chemokines by the signal transducers and activators of transcription 1 (STAT1) pathway (44, 56-58). This complex molecular response leads to attraction of immune cells, which will release more proinflammatory cytokines, such as IFN-γ, IL-1β, and TNF. Local inflammation and activation of antiviral defenses seek to eradicate infection and trigger apoptosis of infected cells. However, in some genetically susceptible individuals, this defense system fails to work properly, instead inducing excessive, progressive inflammation and prolonged death of beta-cells, thus predisposing to the development of T1DM (4).

In this context, high levels of type 1 IFN are found in the pancreas of patients with T1DM (4), and IFN-α is known to contribute to the development of experimental viral-induced diabetes in mice (59, 60). However, recent data demonstrate that type 1 IFN production via TLR3 is critical to prevent diabetes caused by a virus with preferential tropism for pancreatic beta-cells in NOD mouse, suggesting that viral infection and type 1 IFN responses may promote T cell tolerance by enhancing T regulatory cell function and up-regulation of inhibitory molecules and proinflammatory cytokines (53). It is known that the dsRNA-synthetic analog Poly(I:C) can accelerate diabetes in animal models, indicating that dsRNA sensors, such as TLR3, RIG-I and MDA5, can promote diabetes in an IFN-dependent manner (61, 62). Accordingly, human pancreatic islets infected with Coxsackie B5 virus or exposed to IFN-α or IFN-γ

+ IL-1β exhibit increased expression of TLR3, RIG-I and MDA5 (63, 64). Both intracellular and extracellular dsRNA may bind to TLR3 and trigger production of proinflammatory cytokines and chemokines, resulting in beta-cell apoptosis through the activation of NF-κB and IRF-3 (65-67).

Nevertheless, although TLR3 was the first dsRNA sensor identified as being able to activate NF-κB and IRF-3, its role as a primary antiviral receptor was recently called into question (68). *In vivo* antiviral responses against a wide range of viral pathogens were similar in wild-type and *TLR3* knockout mice (69). Indeed, more recent studies show that, whereas NF-κB and IRF-3 activation by extracellular dsRNA is TLR3-dependent, activation by intracellular dsRNA, a product of viral replication in the cytoplasm, also occurs through activation of RIG-I and IFIH1/MDA5 (65, 66). These data indicate that a multiplicity of viral sensors seems to be essential for effective type 1 IFN response in beta-cells and other tissues. Distinct receptors differ in their specificity for viral products. Moreover, distinct viral receptors may differ in their tissue distribution or might be expressed at different time-points during infections. MDA5 is an IFN-induced protein, whereas TLR3 is constitutively expressed; therefore, TLR3-mediated antiviral responses might occur earlier than MDA5-mediated responses (53).

In brief, TLR3 is a plausible candidate for study in T1DM, because it reacts to viral products and is expressed in the pancreas and on antigen-presenting dendritic cells. Accordingly, Eleftherohorinou *et al.* (70) developed a novel pathway-based method to assess the combined effect of more than 20,000 SNPs acting within 84 pathways associated with the innate and acquired immune responses to pathogens, and applied it to data from 14,000 subjects from United Kingdom with 7 common diseases, including T1DM. Variants responsible for the pathway association were identified and used to calculate predictive models for the diseases. These models were tested on an

independent cohort from Northern Finland. They showed that multiple inflammatory pathways, containing 205 SNPs, were associated with T1DM. These SNPs, including SNPs in the TLR3 pathway, were found to be highly predictive of T1DM [91% AUC (area under the receiver operating curve) in the UK sample and 79% AUC in the Finnish cohort].

In addition, Pirie *et al.* (54) reported that of nine *TLR3* polymorphisms studied in a small sample of 153 subjects of Zulu descent from South Africa, a significant association with risk for T1DM was found for the major allele in the 2593 C/T (rs5743313) polymorphism and for the minor alleles in the 2642 C/A (rs5743315) and 2690 A/G (novel) polymorphisms, which were found to be in complete linkage disequilibrium. However, correction of the P-values for the number of comparisons rendered the results no longer significant. Thus, further studies are urgently needed to investigate the association between *TLR3* polymorphisms and T1DM in different populations.

CONCLUSIONS

T1DM is a multifactorial disease associated with both genetic and environmental factors, and it is well known that viral infections play an important role in the development of T1DM in individuals with genetic predisposition. TLR3 recognizes viral replication-derived dsRNA, triggering the release of proinflammatory cytokines by immune system cells. This local inflammation and activation of antiviral defenses seeks to eradicate infection and trigger apoptosis of virus-infected cells. However, in some genetically susceptible individuals, this defense system fails to work properly, instead inducing excessive, progressive inflammation and prolonged death of beta-cells and

thus predisposing to the development of T1DM. Hence, *TLR3* is a good candidate gene for T1DM. However, although several studies have contributed to the understanding of the mechanisms underlying viral infection and TLR3 activity in T1DM pathogenesis, there is just one study reporting association between T1DM and *TLR3* gene polymorphisms. Thus, robust genetic studies are necessary in order to give a better understanding of the role of the *TLR3* gene in the susceptibility for T1DM. Knowledge of the factors associated with T1DM development will enable a keener understanding of its pathogenesis and may provide more effective approaches for the treatment and prevention of this disease.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

REFERENCES

1. Gillespie KM. Type 1 diabetes: pathogenesis and prevention. CMAJ. 2006;175(2):165-70.
2. van Belle TL, Coppieters KT, von Herrath MG. Type 1 diabetes: etiology, immunology, and therapeutic strategies. Physiol Rev. 2011;91(1):79-118.
3. Association AD. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2013;36 Suppl 1:S67-74.
4. Eizirik DL, Colli ML, Ortis F. The role of inflammation in insulitis and beta-cell loss in type 1 diabetes. Nat Rev Endocrinol. 2009;5(4):219-26.
5. Jahromi MM, Eisenbarth GS. Cellular and molecular pathogenesis of type 1A diabetes. Cell Mol Life Sci. 2007;64(7-8):865-72.
6. Kim MS, Polychronakos C. Immunogenetics of type 1 diabetes. Horm Res. 2005;64(4):180-8.
7. Steck AK, Rewers MJ. Genetics of type 1 diabetes. Clin Chem. 2011;57(2):176-85.
8. Todd JA, Walker NM, Cooper JD, Smyth DJ, Downes K, Plagnol V, et al. Robust associations of four new chromosome regions from genome-wide analyses of type 1 diabetes. Nat Genet. 2007;39(7):857-64.
9. Maahs DM, West NA, Lawrence JM, Mayer-Davis EJ. Epidemiology of type 1 diabetes. Endocrinol Metab Clin North Am. 2010;39(3):481-97.
10. Coppieters KT, Boettler T, von Herrath M. Virus infections in type 1 diabetes. Cold Spring Harb Perspect Med. 2012;2(1):a007682.
11. Knip M, Veijola R, Virtanen SM, Hyöty H, Vaarala O, Akerblom HK. Environmental triggers and determinants of type 1 diabetes. Diabetes. 2005;54 Suppl 2:S125-36.
12. Takeda K, Akira S. Toll-like receptors in innate immunity. Int Immunol. 2005;17(1):1-14.
13. Meylan E, Tschopp J, Karin M. Intracellular pattern recognition receptors in the host response. Nature. 2006;442(7098):39-44.
14. Kumar H, Kawai T, Akira S. Pathogen recognition in the innate immune response. Biochem J. 2009;420(1):1-16.
15. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010;140(6):805-20.
16. Booth J, Wilson H, Jimbo S, Mutwiri G. Modulation of B cell responses by Toll-like receptors. Cell Tissue Res. 2011;343(1):131-40.
17. Arancibia SA, Beltran CJ, Aguirre IM, Silva P, Peralta AL, Malinarich F, et al. Toll-like receptors are key participants in innate immune responses. Biol Res. 2007;40(2):97-112.
18. Zarember KA, Godowski PJ. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. J Immunol. 2002;168(2):554-61.
19. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature. 2001;413(6857):732-8.
20. Zipris D. Epidemiology of type 1 diabetes and what animal models teach us about the role of viruses in disease mechanisms. Clin Immunol. 2009;131(1):11-23.

21. Akerblom HK, Vaarala O, Hyoty H, Ilonen J, Knip M. Environmental factors in the etiology of type 1 diabetes. Am J Med Genet. 2002;115(1):18-29.
22. Lien E, Zipris D. The role of Toll-like receptor pathways in the mechanism of type 1 diabetes. Curr Mol Med. 2009;9(1):52-68.
23. Filippi CM, von Herrath MG. Viral trigger for type 1 diabetes: pros and cons. Diabetes. 2008;57(11):2863-71.
24. Hyoty H, Hiltunen M, Knip M, Laakkonen M, Vahasalo P, Karjalainen J, et al. A prospective study of the role of coxsackie B and other enterovirus infections in the pathogenesis of IDDM. Childhood Diabetes in Finland (DiMe) Study Group. Diabetes. 1995;44(6):652-7.
25. Oikarinen M, Tauriainen S, Honkanen T, Oikarinen S, Vuori K, Kaukinen K, et al. Detection of enteroviruses in the intestine of type 1 diabetic patients. Clin Exp Immunol. 2008;151(1):71-5.
26. Elshebani A, Olsson A, Westman J, Tuvemo T, Korsgren O, Frisk G. Effects on isolated human pancreatic islet cells after infection with strains of enterovirus isolated at clinical presentation of type 1 diabetes. Virus Res. 2007;124(1-2):193-203.
27. Yeung WC, Rawlinson WD, Craig ME. Enterovirus infection and type 1 diabetes mellitus: systematic review and meta-analysis of observational molecular studies. BMJ. 2011;342:d35.
28. Jaidane H, Hober D. Role of coxsackievirus B4 in the pathogenesis of type 1 diabetes. Diabetes Metab. 2008;34(6 Pt 1):537-48.
29. Tauriainen S, Oikarinen S, Oikarinen M, Hyoty H. Enteroviruses in the pathogenesis of type 1 diabetes. Semin Immunopathol.33(1):45-55.
30. Banatvala JE, Bryant J, Schernthaner G, Borkenstein M, Schober E, Brown D, et al. Coxsackie B, mumps, rubella, and cytomegalovirus specific IgM responses in patients with juvenile-onset insulin-dependent diabetes mellitus in Britain, Austria, and Australia. Lancet. 1985;1(8443):1409-12.
31. Yoon JW, Austin M, Onodera T, Notkins AL. Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. N Engl J Med. 1979;300(21):1173-9.
32. Dotta F, Censini S, van Halteren AG, Marselli L, Masini M, Dionisi S, et al. Coxsackie B4 virus infection of beta cells and natural killer cell insulitis in recent-onset type 1 diabetic patients. Proc Natl Acad Sci U S A. 2007;104(12):5115-20.
33. Bach JF, Chatenoud L. The hygiene hypothesis: an explanation for the increased frequency of insulin-dependent diabetes. Cold Spring Harb Perspect Med. 2012;2(2):a007799.
34. Serreze DV, Ottendorfer EW, Ellis TM, Gauntt CJ, Atkinson MA. Acceleration of type 1 diabetes by a coxsackievirus infection requires a preexisting critical mass of autoreactive T-cells in pancreatic islets. Diabetes. 2000;49(5):708-11.
35. Tracy S, Drescher KM. Coxsackievirus infections and NOD mice: relevant models of protection from, and induction of, type 1 diabetes. Ann N Y Acad Sci. 2007;1103:143-51.
36. Kariko K, Ni H, Capodici J, Lamphier M, Weissman D. mRNA is an endogenous ligand for Toll-like receptor 3. J Biol Chem. 2004;279(13):12542-50.
37. Amarante MK, Watanabe MA. Toll-like receptor 3: involvement with exogenous and endogenous RNA. Int Rev Immunol. 2010;29(6):557-73.
38. Chang JH, McCluskey PJ, Wakefield D. Toll-like receptors in ocular immunity and the immunopathogenesis of inflammatory eye disease. Br J Ophthalmol. 2006;90(1):103-8.
39. Kaisho T, Akira S. Toll-like receptor function and signaling. J Allergy Clin Immunol. 2006;117(5):979-87; quiz 88.

40. Kawai T, Akira S. Toll-like receptor and RIG-I-like receptor signaling. Ann N Y Acad Sci. 2008;1143:1-20.
41. Moresco EM, LaVine D, Beutler B. Toll-like receptors. Curr Biol. 2011;21(13):R488-93.
42. Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S. Host innate immune receptors and beyond: making sense of microbial infections. Cell Host Microbe. 2008;3(6):352-63.
43. Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity. 2011;34(5):637-50.
44. Khoo JJ, Forster S, Mansell A. Toll-like receptors as interferon-regulated genes and their role in disease. J Interferon Cytokine Res. 2011;31(1):13-25.
45. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124(4):783-801.
46. Yokota S, Okabayashi T, Fujii N. The battle between virus and host: modulation of Toll-like receptor signaling pathways by virus infection. Mediators Inflamm. 2010;2010:184328.
47. Kawai T, Akira S. TLR signaling. Cell Death Differ. 2006;13(5):816-25.
48. Medzhitov R. Recognition of microorganisms and activation of the immune response. Nature. 2007;449(7164):819-26.
49. Bjørnvold M, Munthe-Kaas MC, Egeland T, Joner G, Dahl-Jørgensen K, Njølstad PR, et al. A TLR2 polymorphism is associated with type 1 diabetes and allergic asthma. Genes Immun. 2009;10(2):181-7.
50. Park Y, Park S, Yoo E, Kim D, Shin H. Association of the polymorphism for Toll-like receptor 2 with type 1 diabetes susceptibility. Ann N Y Acad Sci. 2004;1037:170-4.
51. Cooper JD, Walker NM, Smyth DJ, Downes K, Healy BC, Todd JA. Follow-up of 1715 SNPs from the Wellcome Trust Case Control Consortium genome-wide association study in type I diabetes families. Genes Immun. 2009;10 Suppl 1:S85-94.
52. Yu M, Levine SJ. Toll-like receptor, RIG-I-like receptors and the NLRP3 inflammasome: key modulators of innate immune responses to double-stranded RNA viruses. Cytokine Growth Factor Rev. 2011;22(2):63-72.
53. Swiecki M, McCartney SA, Wang Y, Colonna M. TLR7/9 versus TLR3/MDA5 signaling during virus infections and diabetes. J Leukoc Biol. 2011;90(4):691-701.
54. Pirie FJ, Pegoraro R, Motala AA, Rauff S, Rom L, Govender T, et al. Toll-like receptor 3 gene polymorphisms in South African Blacks with type 1 diabetes. Tissue Antigens. 2005;66(2):125-30.
55. Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. Nat Immunol. 2003;4(2):161-7.
56. Yamamoto M, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K, et al. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. J Immunol. 2002;169(12):6668-72.
57. Sato S, Sugiyama M, Yamamoto M, Watanabe Y, Kawai T, Takeda K, et al. Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. J Immunol. 2003;171(8):4304-10.

58. Taniguchi T, Takaoka A. The interferon-alpha/beta system in antiviral responses: a multimodal machinery of gene regulation by the IRF family of transcription factors. Curr Opin Immunol. 2002;14(1):111-6.
59. Stewart TA, Hultgren B, Huang X, Pitts-Meek S, Hully J, MacLachlan NJ. Induction of type I diabetes by interferon-alpha in transgenic mice. Science. 1993;260(5116):1942-6.
60. Alba A, Puertas MC, Carrillo J, Planas R, Ampudia R, Pastor X, et al. IFN beta accelerates autoimmune type 1 diabetes in nonobese diabetic mice and breaks the tolerance to beta cells in nondiabetes-prone mice. J Immunol. 2004;173(11):6667-75.
61. Moriyama H, Wen L, Abiru N, Liu E, Yu L, Miao D, et al. Induction and acceleration of insulitis/diabetes in mice with a viral mimic (polyinosinic-polycytidylic acid) and an insulin self-peptide. Proc Natl Acad Sci U S A. 2002;99(8):5539-44.
62. Devendra D, Jasinski J, Melanitou E, Nakayama M, Li M, Hensley B, et al. Interferon-alpha as a mediator of polyinosinic:polycytidylic acid-induced type 1 diabetes. Diabetes. 2005;54(9):2549-56.
63. Hultcrantz M, Huhn MH, Wolf M, Olsson A, Jacobson S, Williams BR, et al. Interferons induce an antiviral state in human pancreatic islet cells. Virology. 2007;367(1):92-101.
64. Ylipaasto P, Kutlu B, Rasilainen S, Rasschaert J, Salmela K, Teerijoki H, et al. Global profiling of coxsackievirus- and cytokine-induced gene expression in human pancreatic islets. Diabetologia. 2005;48(8):1510-22.
65. Rasschaert J, Ladrière L, Urbain M, Dogusan Z, Katabua B, Sato S, et al. Toll-like receptor 3 and STAT-1 contribute to double-stranded RNA+ interferon-gamma-induced apoptosis in primary pancreatic beta-cells. J Biol Chem. 2005;280(40):33984-91.
66. Dogusan Z, Garcia M, Flamez D, Alexopoulou L, Goldman M, Gysemans C, et al. Double-stranded RNA induces pancreatic beta-cell apoptosis by activation of the toll-like receptor 3 and interferon regulatory factor 3 pathways. Diabetes. 2008;57(5):1236-45.
67. Liu D, Cardozo AK, Darville MI, Eizirik DL. Double-stranded RNA cooperates with interferon-gamma and IL-1 beta to induce both chemokine expression and nuclear factor-kappa B-dependent apoptosis in pancreatic beta-cells: potential mechanisms for viral-induced insulitis and beta-cell death in type 1 diabetes mellitus. Endocrinology. 2002;143(4):1225-34.
68. Meylan E, Tschopp J. Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses. Mol Cell. 2006;22(5):561-9.
69. Edelmann KH, Richardson-Burns S, Alexopoulou L, Tyler KL, Flavell RA, Oldstone MB. Does Toll-like receptor 3 play a biological role in virus infections? Virology. 2004;322(2):231-8.
70. Eleftherohorinou H, Wright V, Hoggart C, Hartikainen AL, Jarvelin MR, Balding D, et al. Pathway analysis of GWAS provides new insights into genetic susceptibility to 3 inflammatory diseases. PLoS ONE. 2009;4(11):e8068.

Table 1. Pathogens-associated molecular pattern (PAMPs) detection by toll-like receptors (TLRs).

TLRs	Localization	Microorganism	PAMPs recognized	Other PRRs involved in recognition
TLR1	Plasma membrane	Bacteria	Triacyl lipoprotein	
TLR2	Plasma membrane	Bacteria	Lipoprotein	
		Fungus	Zymosan, beta-glucan	Dectin-1, NALP-3
		Parasites	TgpI-mutin (*Trypanosoma* sp)	
TLR2/1 TLR2/6	Plasma membrane	Lipoproteins, LTA, PGN, lipoarabinomannan	Bacteria, mycobacteria	NOD1, NOD2, NALP3, NALP1
TLR3	Endolysossome	Viruses	dsRNA	RIG-1, MDA5, NALP3
TRL4	Plasma membrane	Bacteria	LPS	
		Viruses	Structural protein	
		Parasite	Glycoinositolphospholipids (*Trypanosoma* sp)	
		Fungus	Mannan	
TLR5	Plasma membrane	Bacteria	Flagelin	IPAF, NAIP5
TLR6	Plasma membrane	Bacteria	Diacyl lipoprotein	
		Viruses	Diacyl lipoprotein	

		Fungus	Zymosan, beta-glucan	Dectin-1, NALP-3
TLR7	Endolysossome	Viruses	ssRNA	RIG-1, MDA5, NALP3
		Bacteria	RNA	NALP3
		Self		
		Fungus	RNA	
TLR8	Endolysossome	Viruses	RNA	RIG-1, MDA5, NALP3
TLR9	Endolysossome	Viruses	CpG-DNA	AIM2, DAÍ, IFI16
		Bacteria	DNA	AIM2
		Fungus	DNA	
		Parasites	Hemozoin (*Plasmodium* sp)	NALP3
TLR10	Endolysossome	Unknown	Unknown	
TLR11	Plasma membrane	Protozoa	Profilin-like molecule	Unknown

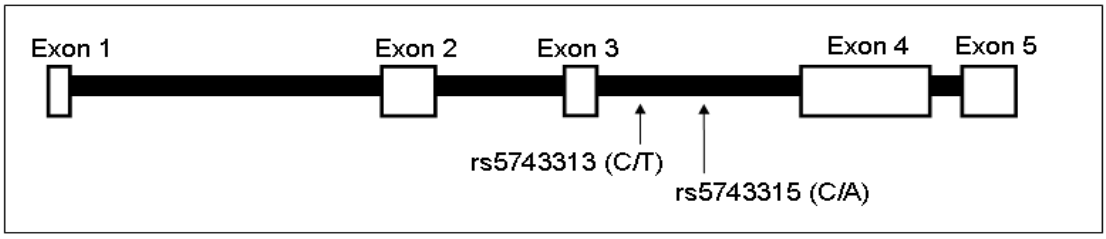

Figure 1. Map of Toll-like receptor (*TLR3*) locus on chromosome 4 (region 4q35). The five exons (boxes) are numbered from left to right according to the transcriptional region. The vertical arrows show the main common polymorphisms associated with type 1 diabetes mellitus.

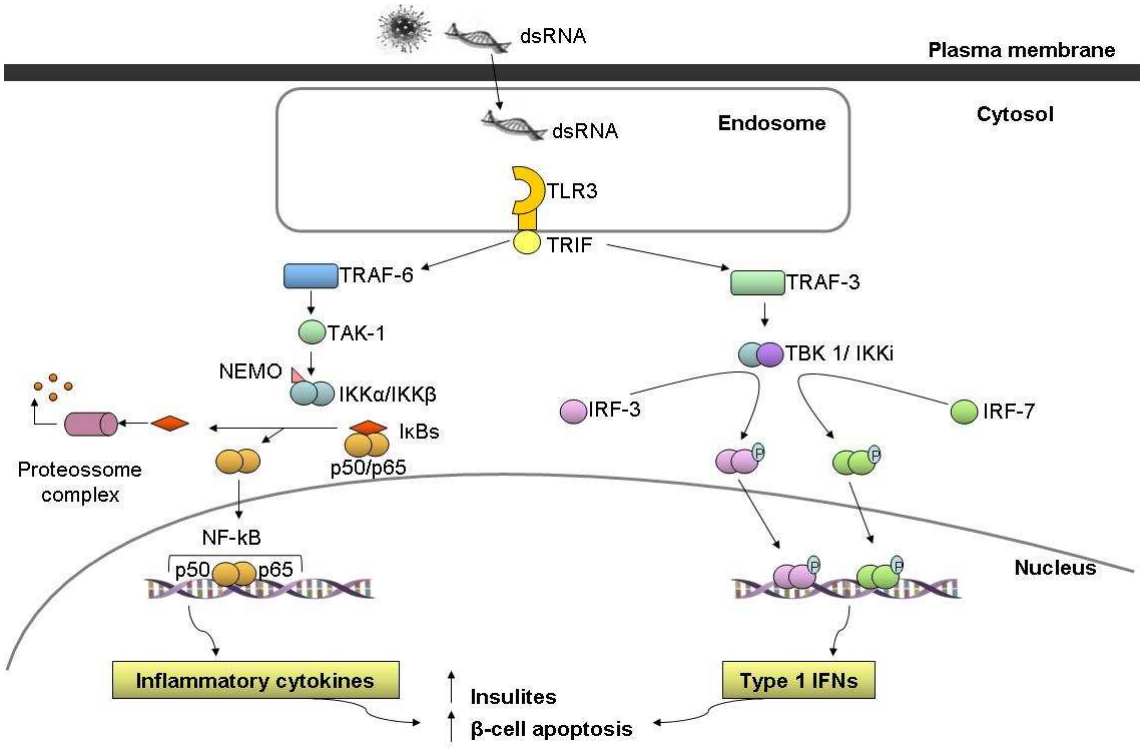

Figure 2. Toll-like receptor 3 (TLR3) signaling in pancreatic beta-cells. Viral double-stranded RNA (dsRNA) binds TLR3 with the resultant recruitment of the adaptor molecule TRIF. Then, TRIF initiates two pathways via TRAF-6 and TRAF-3. TRAF-6 activates TAK1. Activated TAK1 activates the IKK complex, which activates NF-*κ*B subunits, which translocate to the nucleus. In the nucleus, NF-*κ*B triggers the transcription of several genes coding for proinflammatory cytokines and chemokines. TRAF3 activates TBK1/IKKi, which phosphorylate the transcription factors IRF-3 and IRF-7. After phosphorylation, activation and dimerization, IRF-3 and IRF-7, translocate to the nucleus and induce the expression of type 1 interferons, which, after being secreted, will also activate the expression of proinflammatory cytokines and chemokines. This complex molecular response leads to attraction of immune cells, which will release more proinflammatory cytokines, such as IFN-γ, IL-1β, and TNF, and might lead to the beta-cell apoptosis. Adapted from Rasschaert *et al.* (65) and Kumar *et al.* (14).

ARTIGO ORIGINAL

POLIMORFISMOS NO GENE DO RECEPTOR DO TIPO TOLL 3 (*TLR3*) ESTÃO ASSOCIADOS COM RISCO PARA O DIABETES MELLITUS TIPO 1

Polymorphisms in the toll-like receptor 3 *(TLR3)* gene are associated with risk to type 1 diabetes mellitus

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Short title: *TLR3* polymorphisms are associated with type 1 diabetes

ABSTRACT

Introduction Viral pathogens seem to play a major role in triggering the autoimmune destruction that leads to the development of type 1 diabetes mellitus (T1DM). Toll-like receptor 3 (TLR3) has been shown to recognize double-stranded RNA, a molecular signature of most viruses. It is expressed at high levels in pancreatic beta-cells and immune cells, suggesting a role for it in the pathogenesis of T1DM. Therefore, the aim of this study was to investigate whether *TLR3* polymorphisms are associated with T1DM.

Methods Frequencies of the *TLR3* rs5743313, rs11721827, rs3775291, rs13126816, and rs7668666 polymorphisms were analyzed in 476 T1DM patients and in 507 non-diabetic subjects from Brazil. Haplotypes constructed from the combination of these polymorphisms were inferred using a Bayesian statistical method.

Results The rs3775291 and rs13126816 polymorphisms were associated with T1DM in different inheritance models, with the strongest association being observed for the additive model (OR=2.3; 95% CI 1.3-4.1, and OR=2.1, 95% CI 1.4-3.2, respectively). The other three polymorphisms were not significantly associated with T1DM. However, the prevalence of T1DM was higher as more risk alleles of the five polymorphisms were present (P trend=0.002). Moreover, in T1DM patients, the minor alleles of the rs5743313 and rs117221827 polymorphisms were associated with an early age at diagnosis and worse glycemic control.

Conclusion The *TLR3* rs3775291 and rs13126816 polymorphisms are associated with risk for T1DM in Brazilian subjects, while the rs5743313 and rs11721827 polymorphisms are associated with age at T1DM diagnosis and worst glycemic control. The number of risk alleles of the five *TLR3* polymorphisms in the haplotypes seems to

influence the risk for T1DM, suggesting that these polymorphisms might interact in the susceptibility for the disease.

Keywords: Type 1 Diabetes, Toll-like receptor 3, DNA polymorphisms, Haplotype.

INTRODUCTION

Type 1 diabetes mellitus (T1DM) is characterized by severe autoimmune destruction of insulin-producing beta-cells in the pancreas by T lymphocytes and macrophages infiltrating the islets of Langerhans, which renders subjects insulin-dependent for life (1, 2). Several epidemiological, experimental and clinical studies indicate that environmental factors, such as viral pathogens, trigger the autoimmune destruction of beta-cells in genetically susceptible subjects (3-6).

The most well documented correlation between a virus and T1DM has been for enteroviruses, such as Coxsackievirus B (CV-B), which exhibit specific tropism for the pancreas (4, 6-8). Serological and PCR analyses have shown that CV-B is expressed more frequently in T1DM patients than in healthy subjects (9). One CV-B4 strain was isolated from the pancreas of a deceased diabetic child, and this virus was able to induce diabetes when inoculated in a susceptible mouse strain (10). Moreover, CV-B4 was identified in the pancreatic tissue from three of six T1DM patients (11), and it was capable of infecting human islet *in vitro*, impairing glucose-stimulated insulin secretion (12). The pathogenic role of enteroviruses in T1DM seems to involve damage to beta-cells and local induction of proinflammatory mediators (13).

The immune response to virus infection begins with the recognition of pathogen-associated molecular patterns (PAMPs) as “nonself” signatures. This recognition occurs through host pattern recognition receptors (PRRs), and triggers intracellular signaling events that induce innate immunity, the first line of defense against microbial infection. Many types of PRRs have been identified so far, including toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). These PRRs recognize specific

PAMPs in different cellular compartments, inducing the expression of proinflammatory cytokines, chemokines and co-stimulatory molecules, which eliminate the pathogens and active pathogen-specific adaptive immune responses (14-16).

Of the PRRs, the most extensively studied are TLRs, which are type I transmembrane glycoproteins originally identified in *Drosophila* (17, 18). Human homologs are known to comprise at least 10 members with different response triggers (14). TLR3 has been shown to recognize double-stranded RNA (dsRNA), a molecular signature of most viruses, and it is expressed at high levels in human and mouse pancreatic beta-cells and in antigen-presenting dendritic cells (13, 19), suggesting a potential role for this receptor in the infectious etiology of T1DM. However, to date, only one study evaluated the association between polymorphisms in the *TLR3* gene and T1DM: Pirie *et al*. (20) reported that of nine *TLR3* polymorphisms studied in a small sample of 153 subjects of Zulu descent (South Africa), a significant association with risk for T1DM was found for the major allele in the rs5743313 (C/T) polymorphism and for the minor alleles in the rs5743315 (C/A) and 2690 A/G (novel) polymorphisms, which were found to be in complete linkage disequilibrium. Nevertheless, correction of the P-values for the number of comparisons rendered the results no longer significant.

Thus, further studies are needed to investigate the association between *TLR3* polymorphisms and T1DM in different populations. Within this context, the aim of this study was to investigate the association of the rs5743313 (C/T), rs3775291 (A/G), rs11721827 (A/C), rs13126816 (A/G) and rs7668666 (A/C) polymorphisms in the *TLR3* gene with T1DM in a white Brazilian population.

MATERIALS AND METHODS

Subjects, phenotype measurements, and laboratory analyses

This was a case-control study designed to investigate whether the *TLR3* rs5743313 (C/T), rs11721827 (A/C), rs3775291 (A/G), rs13126816 (A/G) and rs766866 (A/C) polymorphisms are associated with T1DM. The diabetic sample comprised 476 unrelated patients from the outpatient clinic at the Hospital de Clínicas de Porto Alegre (Rio Grande do Sul, Brazil). Patients were considered to have T1DM if they had been diagnosed with hyperglycemia according to ADA diabetes criteria before the age of 30 years, requires insulin for glycemic control and do not had a clinical profile compatible with other diabetes mellitus subtypes assessed by experienced Endocrinologists (21). The non-diabetic group comprised 507 healthy blood donors who did not have diabetes mellitus or family history for this disease. All subjects were self-defined as white.

A standard questionnaire was used to collect information on age, age at DM diagnosis, and drug treatment and all patients underwent physical and laboratory evaluations. They were weighed unshod, wearing light outdoor clothes and their height was measured. Body mass index (BMI) was calculated as weight (kg)/height square (meters). Blood pressure (BP) was measured by a trained researcher, with a mercury sphygmomanometer on the left arm, using an appropriated cuff size, in a sitting position, after a 5-min rest. The mean of two measurements taken 1 min apart was used to calculate systolic and diastolic BP. Arterial hypertension (AH) was defined as BP levels higher than 140/90 mmHg at initial visit and at two follow-up visits within 1 month of the initial visit, or if the presence of AH was registered on medical records.

Serum and plasma samples were taken after 12 hours of fasting for laboratory analyses. Plasma glucose levels were determined using the glucose oxidase method.

Creatinine levels were determined using the Jaffe reaction and were traceable to IDMS. Glycated hemoglobin (GHb) measurements were performed by different methods and results were traceable to the DCCT (Diabetes Control and Complications Trial) method by off-line calibration or through conversion formulae (22). Total plasma cholesterol, HDL cholesterol and triglycerides were assayed using enzymatic methods.

Genotyping

DNA was extracted from peripheral blood leukocytes by a standardized salting-out procedure (23). *TLR3* polymorphisms were genotyped using primers and probes contained in the Human Custom TaqMan Genotyping Assay 20x (Life Technologies, Foster City, CA, USA), and described in **Table 1**. Reactions were conducted in 384-well plates, in a total 5 µl reaction volume using 2 ng of genomic DNA, TaqMan Genotyping Master Mix 1x (Life Technologies) and Custom TaqMan Genotyping Assay 1x. The plates were then positioned in a real-time PCR thermal cycler (ViiA7 Real Time PCR System; Life Technologies) and heated for 10 min at 95^{o}C, followed by 50 cycles of 95^{o}C for 15 s and 60^{o}C for 1 min. Fluorescence data files from each plate were analyzed using automated allele-calling software (Life Technologies).

The location of the rs11721827 (A/C), rs13126816 (A/G), rs5743313 (C/T), rs7668666 (A/C) and rs3775291 (A/G) polymorphisms at the *TLR3* gene is shown in **Figure 1**. *TLR3* polymorphisms were selected from the International HapMap Project (24). Due to linkage disequilibrium (LD) between some of the common polymorphisms in this gene, at least five polymorphisms had to be genotyped to estimate all haplotypes with more than 5% frequency and that would cover 83% of all possible *TLR3* haplotypes. The rs5743313 and rs3775291 polymorphisms were also selected on the basis of previous studies: Pirie

et al. (20) reported that the rs5734313 polymorphism was associated with T1DM, while Ranjith-Kumar *et at* (25) reported that the rs3775291 polymorphism was functional, being associated with natural resistance to HIV-1 infection (26).

Statistical analysis

Allele frequencies were determined by gene counting, and departures from the Hardy–Weinberg equilibrium (HWE) were verified using χ^2-tests. Allele and genotype frequencies were compared between groups of subjects using χ^2-tests. Between all pairs of biallelic loci, we examined widely used measures of LD, Lewontin's D' |D'| and r^2 (27). The haplotypes constructed from the combination of the five *TLR3* polymorphisms and their frequencies were inferred using the Phase 2.1 program, which implements a Bayesian statistical method (28). We also used this program to compare the distributions of different *TLR3* haplotypes between T1DM patients and non-diabetic subjects through permutation analyses of 10,000 random replicates (28).

Clinical and laboratory characteristics were compared between groups by using unpaired Student's t-test or χ^2, as appropriate. Variables with normal distribution are presented as mean ± SD. Variables with skewed distribution were log-transformed before analyses and are presented as median (minimum-maximum values). The magnitude of the association of different haplotypes with T1DM was estimated using odds ratio (OR) tests with 95% CI. Results for which P was less than 0.05 were considered statistically significant. Bonferroni correction was used to account for multiple comparisons. These statistical analyses were done using SPSS version 18.0 (SPSS, Chicago, IL, USA).

Power calculations (PEPI program, v. 4.0) showed that this study has a power of approximately 80% at a significance level of 0.05 to detect an OR of 1.5 (for the presence of the mutated alleles).

RESULTS

Sample description

The main clinical and laboratory characteristics of the 476 T1DM patients belonging to the present study were as follows: mean age was 33.5 ± 13.3 years; age at T1DM diagnosis was 16 (1 - 30) years; T1DM duration was 15 (1 - 67) years; mean GHb was 8.5 ± 2.0%; and mean BMI was 24.7 ± 4.3 kg/m^2. Males comprised 49.8% of the sample, and 26% of all patients had AH. The mean age of the non-diabetic group was 44.0 ± 7.8 years, and males comprised 55.0% of the sample.

Genotype and allele distributions

Genotype and allele frequencies of the rs5743313 (C/T), rs3775291 (A/G), rs11721827 (A/C), rs13126816 (A/G) and rs7668666 (A/C) polymorphisms of the *TLR3* gene in T1DM patients and non-diabetic subjects are depicted in **Table 2**. All genotypes were in agreement with those predicted by the HWE in non-diabetic subjects (P > 0.05). Distributions of the rs5743313, rs11721827 and rs7668666 polymorphisms did not differ statistically between the two analyzed samples after Bonferroni correction. Moreover, frequencies of these three polymorphisms also did not differ statistically when assuming dominant, recessive or additive inheritance models for the presence of the mutated alleles (all P > 0.05; data not shown).

Both genotype and allele frequencies of the rs3775291 polymorphism were differently distributed between T1DM patients and non-diabetic subjects (P = 0.016 and P = 0.027, respectively); however, being conservative, these frequencies were not statistically different between groups when taking into consideration a Bonferroni threshold of 0.010 (P = 0.05 divided by 5 *TLR3* polymorphisms) (**Table 2**). Interestingly, the G allele of the rs3775291 polymorphism was associated with risk for T1DM under additive and dominant inheritance models (P = 0.006 and P = 0.007, respectively), further supporting a role for the rs3775291 polymorphism on T1DM. It is worth noting that the association was stronger for the additive inheritance model (OR = 2.3; 95% CI 1.3-4.1). Genotype and allele frequencies of the rs13126816 polymorphism differed significantly between T1DM patients and non-diabetic subjects ($P = 1x10^{-3}$ and $P = 1x10^{-5}$, respectively) after Bonferroni corrections (**Table 2**). Furthermore, the rs13126816 G allele was associated with risk for T1DM under dominant, recessive and additive models (P = 0.007, $P = 1x10^{-3}$ and $P = 1x10^{-3}$; respectively), with the strongest association being observed for the additive model (OR = 2.1, 95% CI 1.4-3.2).

Table 3 illustrates the clinical and laboratory characteristics of T1DM patients grouped according to the presence of the risk allele (mutated) of each one of the analyzed *TLR3* polymorphisms. Gender, age, age at T1DM diagnosis, fasting plasma glucose (FPG) and GHb did not differ between groups of patients broken down by the presence of the mutated alleles of the rs3775291, rs13126816 and rs7668666 polymorphisms, taking into account Bonferroni corrections. On the other hand, patients carrying the T allele of the rs5743313 polymorphism had an early age at T1DM diagnosis and a worse glycemic control as compared with patients with the T/T genotype (**Table 3**). In addition, patients carrying the C allele of the rs117221827

polymorphism showed an early age at diagnosis and higher levels of fasting plasma glucose than patients with the A/A genotype.

Haplotype distributions and linkage disequilibrium

We used a Bayesian statistical method to estimate the frequency of different haplotypes produced by the combination of the five *TLR3* polymorphisms in T1DM patients and non-diabetic subjects. Thirty-two haplotypes were inferred in both samples, but only those haplotypes with frequencies higher than 1% are shown in **Table 4**. The most frequent haplotypes were Ht2, Ht4, Ht8, Ht11, Ht12 and Ht24; however, only Ht2, Ht4 and Ht11 had frequencies higher than 10%. Permutation analysis showed that the distributions of all inferred haplotypes were statistically different between T1DM patients and non-diabetic subjects (P = 0.001) because of differences in the frequencies of Ht3, Ht4, Ht9, Ht10, Ht12, and Ht16. While Ht9, Ht10, Ht12 and Ht16 were more frequent in T1DM patients, Ht3 and Ht4 showed the opposite result, being more frequent in non-diabetic subjects (**Table 4**). It is noteworthy that taking into account both |D'| and r^2 measurements, we did not find any significant LD between all pairs of combination of the five analyzed polymorphisms (**Figure 1**).

Figure 2 depicts T1DM patients and non-diabetic subjects broken down according to the presence of the number of risk alleles (mutated) in the estimated haplotypes: a) 0-2; b) 3-4; c) 5-7, and d) 8-10 risk alleles. Interestingly, the prevalence of T1DM is higher as more risk alleles are present in the patients (P-trend = 0.002). Moreover, T1DM patients carrying haplotypes with more than 5 risk alleles of the analyzed polymorphisms had higher levels of fasting plasma glucose than patients with ≤ 4 risk alleles [172.0 (46-603) *vs.* 110.5 (40-276) mg/dl, respectively; P = $1x10^{-3}$]. Age, age at T1DM

diagnosis, gender, and GHb did not differ between these groups after Bonferroni corrections (data not shown).

DISCUSSION

For the first time, the rs13126816 and rs3775291 polymorphisms were significantly associated with risk for T1DM. We also reported an association of the *TLR3* rs5743313 and rs117221827 polymorphisms with age at T1DM diagnosis and glycemic control in T1DM patients.

The ligation of dsRNA to the TLR3 leads to receptor dimerization and to the resultant recruitment of the adaptor molecule, TRIF (TIR-domain-containing adaptor inducing IFN-β), to the cytoplasmic domain of TLR3, known as TIR (toll-IL-1R-resistance). TRIF initiates signaling pathways that activate the downstream transcription factors interferon regulatory factor (IRF)-3 and -7 and nuclear factor (NF)-κB, which in turn trigger the expression and secretion of type I interferons (IFN-I), inflammatory cytokines and chemokines, as well as the maturation of dendritic cells, a key event in the generation of adaptive immunity (16, 29, 30). This local inflammation coupled with triggering of antiviral defenses will in most cases eradicate the viral infection. However, in some genetically susceptible subjects, these cellular attempts to eradicate the infection might go wrong and induce progressive inflammation and prolonged pancreatic beta-cell death; thus, predisposing for T1DM development (13). Accordingly, IFN-I production has been implicated as potential mediators of viral/dsRNA-induced T1DM (31-33).

Viral infections or dsRNA increase the expression of mRNAs encoding for TLR3 and for genes downstream of its signaling pathway in rat and human beta-cells (34-37). Therefore, triggering of TLR3 by a synthetic analog of viral dsRNA, poly(I:C),

precipitates the development of diabetes in rat and mouse models (32, 33, 38-40). However, a recent study suggested that TLR3 is also required to prevent diabetes in mice infected with encephalomyocarditis virus strain D, which has tropism for beta-cells (41). Infection of *Tlr3*$^{-/-}$ mice caused diabetes due to impaired IFN-I responses and virus-induced beta-cell damage rather than T cell-mediated autoimmunity, suggesting that optimal functioning of viral sensors and prompt IFN-I responses are required to prevent diabetes when caused by a virus that infects and damages beta-cells (41).

Interestingly, Alkanani *et al.* (42) reported that activating peripheral blood mononuclear cells (PBMNCs) with TLR3, TLR4 or TLR7/8 agonists *in vitro* led to dysregulated IL-1β and IL-6 pathways in monocytes and myeloid dendritic cells from islet autoantibodies-positive subjects *vs*. seronegative individuals. This dysregulation was more pronounced in children aged <11 years, implying that alterations in the innate immune system are detectable in genetically susceptible individuals and could be linked with the early course of T1DM. Eleftherohorinou *et al.* (43) developed a novel pathway-based method to assess the combined effect of more than 20,000 single nucleotide polymorphisms (SNPs) acting within 84 pathways associated with innate and acquired immune responses to pathogens, and applied it to data from 14,000 subjects from United Kingdom with 7 common diseases, including T1DM. They showed that multiple inflammatory pathways, containing 205 SNPs, were associated with T1DM. These SNPs, including SNPs in genes activated by TLR3, were found to be highly predictive of T1DM.

Within this context, *TLR3* is a plausible candidate gene for T1DM susceptibility. Thus, here, we analyzed the association of five *TLR3* polymorphisms with T1DM. Frequencies of the *TLR3* rs5743313, rs11721827 and rs7668666 polymorphisms were not significantly different between T1DM patients and non-diabetic subjects; however,

the rs13126816 and rs3775291 polymorphisms were significantly associated with risk for T1DM, probably under an additive inheritance model. Interestingly, the prevalence of T1DM was higher as more risk alleles of the five analyzed polymorphisms were present in the *TLR3* haplotypes.

As already mentioned, in addition to the present study, only the study of Pirie *et al.* (20) evaluated the association between polymorphisms in the *TLR3* gene and T1DM. These authors reported that the major allele in the rs5743313 polymorphism and the minor alleles in the rs5743315 and 2690 A/G polymorphisms were associated with risk for T1DM; nevertheless, probably due to the small sample size analyzed, this association was not maintained after correction for multiple comparisons. We were not able to evaluate the rs5743315 (C/A) polymorphism in our population because only the C allele is found in Europeans (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=5743315). The novel polymorphism (2690 A/G) described by Pirie *et al*. (20) is in complete LD with the rs5743315 polymorphism and, as a result, its minor allele will also probably not be found in Europeans. The rs5743313 polymorphism was analyzed in both studies, with our data indicating no association between this polymorphism and T1DM, while the results of Pirie *et al*. (20) suggest a weak association with this disease. These contradictory results may be explained by ethnic differences between the two populations since distributions of the *TLR3* polymorphisms are clearly different between African and European or Caucasian populations.

In the present study, for the first time, we reported an association of the *TLR3* rs5743313 and rs117221827 polymorphisms with age at T1DM diagnosis and glycemic control in T1DM patients. Furthermore, T1DM patients carrying *TLR3* haplotypes with more than 5 risk alleles of the analyzed polymorphisms had significantly higher levels

of fasting plasma glucose than patients with ≤ 4 risk alleles. Further studies are needed to confirm this data and also to clarify how these polymorphisms influence age at T1DM diagnosis and glycemic control.

The exact mechanisms by which *TLR3* polymorphisms contribute to T1DM pathogenesis remain yet to be explored. The rs5743313, rs11721827, rs13126816 and rs7668666 polymorphisms are intronic, and probably are not real disease-causing variants, but could be simply reflecting the effects of a functional variant located elsewhere in the *TLR3* gene. However, it is also known that introns may contain regulatory sequences and even encode RNA with regulatory function or a protein. The rs3775291 polymorphism in exon 4 results in amino acid substitution (Leu412Phe), affecting a residue in the hydrophobic core of the TLR3 extracellular domain (ectodomain – ECD) that is absolutely conserved in all species from mammals to fish (25). The TLR3 ECD has a horseshoe-like shape, characteristic of multiple leucine-rich repeats (LRR), that possibly increases the available surface for dsRNA binding in this region (44). Ranjith-Kumar *et al*. (25) reported that the 412Phe (G) allele causes a 30% loss of activity of the TLR3 in response to its ligands, presumably by destabilizing the LRR architecture. Moreover, another *in vitro* study showed that TLR3 molecules carrying the 412Phe allele allow increased CV-B replication compared with Leu412 receptors (45). However, Sironi *et al*. (26) reported that PBMNCs from individuals carrying the 412Phe allele sustained lower HIV-1 replication compared with that of Leu/Leu homozygotes. Although further studies are necessary to evaluate the effect of the rs3775291 (Leu412Phe) in the activity of TLR3 in pancreatic beta-cells, we hypothesized that during infection with a beta-cell tropic virus, the attenuated activity of the TLR3 412Phe mutant in hematopoietic cells may lead to blunted IFN-I response, uncontrolled virus replication and, consequently, T1DM. This hypothesis is in

agreement with the results of McCartney *et al*. (41), showing that infection in *Tlr3*$^{-/-}$ mice caused diabetes due to impaired IFN-I responses and virus-induced beta-cell damage.

Some factors could have interfered with the findings of the present study. First, we cannot rule out the possibility of population stratification bias when analyzing our samples, even though only white subjects were studied and both T1DM patients and non-diabetic subjects were recruited from the same hospital, thus reducing the risk of false-positive/negative associations due to this bias. Second, we cannot fully exclude the possibility of a type II error when analyzing the association between the analyzed polymorphisms and T1DM. Even though our power to detect an OR of 1.5 was approximately 80% for all analyzed polymorphisms, we cannot fully exclude the possibility that the *TLR3* rs5743313, rs11721827 and rs7668666 polymorphisms would be associated with T1DM with lower ORs. Additional genetic studies and meta-analysis are needed in order to confirm the association of *TLR3* polymorphisms with risk for T1DM in different ethnicities.

In conclusion, the results of this study indicate that the *TLR3* rs3775291 and rs13126816 polymorphisms are associated with risk for T1DM in white Brazilians subjects. Moreover, the prevalence of this disease increases as more risk alleles of the five analyzed polymorphisms are present in the *TLR3* haplotypes, indicating that they might interact in the susceptibility for T1DM. The *TLR3* rs11721827 and rs5743313 polymorphisms seem to be associated with an early age at T1DM diagnosis and a worse glycemic control in our population.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

BIBLIOGRAFIA

1. Gillespie KM. Type 1 diabetes: pathogenesis and prevention. CMAJ. 2006 Jul;175(2):165-70.
2. van Belle TL, Coppieters KT, von Herrath MG. Type 1 diabetes: etiology, immunology, and therapeutic strategies. Physiol Rev. 2011 Jan;91(1):79-118.
3. Maahs DM, West NA, Lawrence JM, Mayer-Davis EJ. Epidemiology of type 1 diabetes. Endocrinol Metab Clin North Am. 2010 Sep;39(3):481-97.
4. Coppieters KT, Boettler T, von Herrath M. Virus infections in type 1 diabetes. Cold Spring Harb Perspect Med. 2012 Jan;2(1):a007682.
5. Knip M, Veijola R, Virtanen SM, Hyöty H, Vaarala O, Akerblom HK. Environmental triggers and determinants of type 1 diabetes. Diabetes. 2005 Dec;54 Suppl 2:S125-36.
6. Jaidane H, Hober D. Role of coxsackievirus B4 in the pathogenesis of type 1 diabetes. Diabetes Metab. 2008 Dec;34(6 Pt 1):537-48.
7. Filippi CM, von Herrath MG. Viral trigger for type 1 diabetes: pros and cons. Diabetes. 2008 Nov;57(11):2863-71.
8. Tauriainen S, Oikarinen S, Oikarinen M, Hyoty H. Enteroviruses in the pathogenesis of type 1 diabetes. 2011 Semin Immunopathol. Jan;33(1):45-55.
9. Zipris D. Epidemiology of type 1 diabetes and what animal models teach us about the role of viruses in disease mechanisms. Clin Immunol. 2009 Apr;131(1):11-23.
10. Yoon JW, Austin M, Onodera T, Notkins AL. Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. N Engl J Med. 1979 May 24;300(21):1173-9.
11. Dotta F, Censini S, van Halteren AG, Marselli L, Masini M, Dionisi S, et al. Coxsackie B4 virus infection of beta cells and natural killer cell insulitis in recent-onset type 1 diabetic patients. Proc Natl Acad Sci U S A. 2007 Mar 20;104(12):5115-20.
12. Elshebani A, Olsson A, Westman J, Tuvemo T, Korsgren O, Frisk G. Effects on isolated human pancreatic islet cells after infection with strains of enterovirus isolated at clinical presentation of type 1 diabetes. Virus Res. 2007 Mar;124(1-2):193-203.
13. Eizirik DL, Colli ML, Ortis F. The role of inflammation in insulitis and beta-cell loss in type 1 diabetes. Nat Rev Endocrinol. 2009 Apr;5(4):219-26.
14. Kumar H, Kawai T, Akira S. Pathogen recognition in the innate immune response. Biochem J. 2009 May 15;420(1):1-16.
15. Wilkins C, Gale M, Jr. Recognition of viruses by cytoplasmic sensors. Curr Opin Immunol. 2010 Feb;22(1):41-7.
16. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010 Mar;140(6):805-20.
17. Meylan E, Tschopp J, Karin M. Intracellular pattern recognition receptors in the host response. Nature. 2006 Jul 6;442(7098):39-44.
18. Arancibia SA, Beltran CJ, Aguirre IM, Silva P, Peralta AL, Malinarich F, et al. Toll-like receptors are key participants in innate immune responses. Biol Res. 2007;40(2):97-112.
19. Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature. 2001 Oct 18;413(6857):732-8.

20. Pirie FJ, Pegoraro R, Motala AA, Rauff S, Rom L, Govender T, et al. Toll-like receptor 3 gene polymorphisms in South African Blacks with type 1 diabetes. Tissue Antigens. 2005 Aug;66(2):125-30.
21. American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2013 Jan;36 Suppl 1:S67-74.
22. Camargo JL, Zelmanovitz T, Paggi A, Friedman R, Gross JL. Accuracy of conversion formulae for estimation of glycohaemoglobin. Scand J Clin Lab Invest. 1998 Oct;58(6):521-8.
23. Lahiri DK, Nurnberger JI. A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. Nucleic Acids Res. 1991 Oct;19(19):5444.
24. The International HapMap Project. Nature. 2003 Dec 18;426(6968):789-96.
25. Ranjith-Kumar CT, Miller W, Sun J, Xiong J, Santos J, Yarbrough I, et al. Effects of single nucleotide polymorphisms on Toll-like receptor 3 activity and expression in cultured cells. J Biol Chem. 2007 Jun 15;282(24):17696-705.
26. Sironi M, Biasin M, Cagliani R, Forni D, De Luca M, Saulle I, et al. A common polymorphism in TLR3 confers natural resistance to HIV-1 infection. J Immunol. 2012 Jan 15;188(2):818-23.
27. Hedrick PW. Gametic disequilibrium measures: proceed with caution. Genetics. 1987 Oct;117(2):331-41.
28. Stephens M, Smith NJ, Donnelly P. A new statistical method for haplotype reconstruction from population data. Am J Hum Genet. 2001 Apr;68(4):978-89.
29. Kawai T, Akira S. TLR signaling. Cell Death Differ. 2006 May;13(5):816-25.
30. Botos I, Liu L, Wang Y, Segal DM, Davies DR. The toll-like receptor 3:dsRNA signaling complex. Biochim Biophys Acta. 2009 Sep-Oct;1789(9-10):667-74.
31. Huang X, Yuang J, Goddard A, Foulis A, James RF, Lernmark A, et al. Interferon expression in the pancreases of patients with type I diabetes. Diabetes. 1995 Jun;44(6):658-64.
32. Devendra D, Jasinski J, Melanitou E, Nakayama M, Li M, Hensley B, et al. Interferon-alpha as a mediator of polyinosinic:polycytidylic acid-induced type 1 diabetes. Diabetes. 2005 Sep;54(9):2549-56.
33. Lang KS, Recher M, Junt T, Navarini AA, Harris NL, Freigang S, et al. Toll-like receptor engagement converts T-cell autoreactivity into overt autoimmune disease. Nat Med. 2005 Feb;11(2):138-45.
34. Rasschaert J, Ladrière L, Urbain M, Dogusan Z, Katabua B, Sato S, et al. Toll-like receptor 3 and STAT-1 contribute to double-stranded RNA+ interferon-gamma-induced apoptosis in primary pancreatic beta-cells. J Biol Chem. 2005 Oct;280(40):33984-91.
35. Dogusan Z, Garcia M, Flamez D, Alexopoulou L, Goldman M, Gysemans C, et al. Double-stranded RNA induces pancreatic beta-cell apoptosis by activation of the toll-like receptor 3 and interferon regulatory factor 3 pathways. Diabetes. 2008 May;57(5):1236-45.
36. Ylipaasto P, Kutlu B, Rasilainen S, Rasschaert J, Salmela K, Teerijoki H, et al. Global profiling of coxsackievirus- and cytokine-induced gene expression in human pancreatic islets. Diabetologia. 2005 Aug;48(8):1510-22.
37. Hultcrantz M, Huhn MH, Wolf M, Olsson A, Jacobson S, Williams BR, et al. Interferons induce an antiviral state in human pancreatic islet cells. Virology. 2007 Oct 10;367(1):92-101.
38. Sobel DO, Newsome J, Ewel CH, Bellanti JA, Abbassi V, Creswell K, et al. Poly I:C induces development of diabetes mellitus in BB rat. Diabetes. 1992 Apr;41(4):515-20.

39. Moriyama H, Wen L, Abiru N, Liu E, Yu L, Miao D, et al. Induction and acceleration of insulitis/diabetes in mice with a viral mimic (polyinosinic-polycytidylic acid) and an insulin self-peptide. Proc Natl Acad Sci U S A. 2002 Apr 16;99(8):5539-44.
40. Wen L, Peng J, Li Z, Wong FS. The effect of innate immunity on autoimmune diabetes and the expression of Toll-like receptors on pancreatic islets. J Immunol. 2004 Mar;172(5):3173-80.
41. McCartney SA, Vermi W, Lonardi S, Rossini C, Otero K, Calderon B, et al. RNA sensor-induced type I IFN prevents diabetes caused by a β cell-tropic virus in mice. J Clin Invest. 2011 Apr;121(4):1497-507.
42. Alkanani AK, Rewers M, Dong F, Waugh K, Gottlieb PA, Zipris D. Dysregulated Toll-like receptor-induced interleukin-1β and interleukin-6 responses in subjects at risk for the development of type 1 diabetes. Diabetes. 2012 Oct;61(10):2525-33.
43. Eleftherohorinou H, Wright V, Hoggart C, Hartikainen AL, Jarvelin MR, Balding D, et al. Pathway analysis of GWAS provides new insights into genetic susceptibility to 3 inflammatory diseases. PLoS ONE. 2009;4(11):e8068.
44. Choe J, Kelker MS, Wilson IA. Crystal structure of human toll-like receptor 3 (TLR3) ectodomain. Science. 2005 Jul 22;309(5734):581-5.
45. Gorbea C, Makar KA, Pauschinger M, Pratt G, Bersola JL, Varela J, et al. A role for Toll-like receptor 3 variants in host susceptibility to enteroviral myocarditis and dilated cardiomyopathy. J Biol Chem. 2010 Jul 23;285(30):23208-23.

Table 1. Primers and probes used for the genotyping of the analyzed *TLR3* polymorphisms.

Polymorphisms	Primers	TaqMan MGB Probes
rs117221827 (A/C)	F: 5' – TTCCCCACTATTTTTATGTTGCTGTCT -3'	VIC: 5'- AAAACAATTACGAATGGACC – 3'
	R: 5'- GCATCTAACTCTGGAGCTCCAAAAT – 3'	FAM: 5'- ATAAAACAATTACTAATGGACC-3'
rs13126816 (A/G)	F: 5' – GCAACGGAAAAGGCAATCTAGAAGA -3'	VIC: 5'- AACCTCCCATTTTGCTC -3'
	R: 5' – AAGTCTTGAAGTTCAGTGAGCGA- 3'	FAM: 5' – ACCTCCCATCTTGCTC -3'
rs5743313 (C/T)	F: 5'- CATTGGTGTCATCCTCCTGAGA- 3'	VIC: 5' – TCTCCCGACCTCTCC- 3'
	R: 5' – GCAGGGCGGCAGAGT -3'	FAM: 5'- TCTCCCAACCTCTCC- 3'
rs7668666 (A/C)	R: 5' – CTCTGCATTTTCACATACAGGTTTGT- 3'	VIC: 5' – TGTATTTTACACTAATTTTG – 3'
	R: 5' – GGATTTCCAGTAGTCTTATAGCCTGGA- 3'	FAM: 5' – TGTATTTTACACTCATTTTG – 3'
rs3775291 (A/G)	F: 5'- CCAAGAGAAAGCATCACTCTCTATTTTTG- 3'	VIC: 5'- CCCTTACACATATTCAACC-3'
	R: 5' – GTTTGCGAACTTTGACAAATGAAACATT-3'	FAM: 5'- CCTTACACATACTCAACC-3'

Table 2. Genotype and allele frequencies of the *TLR3* polymorphisms in patients with type 1 diabetes mellitus (T1DM) and non-diabetic subjects

	T1DM patients	Non-diabetic subjects	P value*
rs5743313 (C/T)	n= 476	n= 507	
Genotype			
C/C	298 (60.1)	322 (63.5)	0.549
C/T	152 (34.1)	159 (31.4)	
T/T	26 (5.8)	26 (5.1)	
Allele			
C	0.78	0.79	0.778
T	0.22	0.21	
rs11721827 (A/C)	n= 313	n=309	
Genotype			
A/A	225 (71.9)	207 (66.9)	0.055
A/C	68 (21.7)	90 (29.1)	
C/C	20 (6.4)	12 (4.0)	
Allele			
A	0.83	0.82	0.634
C	0.17	0.18	
rs3775291 (A/G)	n= 402	n= 301	
Genotype			
A/A	22 (5.5)	34 (11.3)	0.016
A/G	178 (44.3)	132 (43.9)	
G/G	202 (50.2)	135 (44.8)	
Allele			

A	0.28	0.33	0.027
G	0.72	0.67	
rs13126816 (A/G)	n= 447	n= 422	
Genotype			
A/A	46 (10.3)	71 (16.8)	$1x10^{-3}$
A/G	172 (38.5)	182 (43.1)	
G/G	229 (51.2)	169 (40.1)	
Allele			
A	0.30	0.38	$1x10^{-5}$
G	0.70	0.62	
rs7668666 (A/C)	n= 450	n= 423	
Genotype			
A/A	34 (7.6)	46 (10.9)	0.077
A/C	157 (34.9)	162 (38.3)	
C/C	259 (57.5)	215 (50.8)	
Allele			
A	0.25	0.30	0.022
C	0.75	0.70	

Data are shown as number (%) or proportion. *P values were computed by χ^2-test comparing T1DM patients and non-diabetic subjects. Only P values lower than the Bonferroni threshold (P = 0.010) were considered statistically significant.

Table 3. Clinical and laboratory characteristics of T1DM patients broken down by the presence of the mutated alleles of the analyzed *TLR3* polymorphisms

Polymorphism	**Other genotype**	**Presence of risk allele**	**P value***
rs5743313 (C/T)	**C/C**	**C/T + T/T**	
Gender (% male)	53.5	58.5	0.628
Age (years)	40.6 ± 10.5	36.8 ± 13.8	$1x10^{-3}$
Age at diagnosis (years)	18 (2 - 51)	10 (1 - 43)	$1x10^{-10}$
FPG (mg/dl)	111.5 (38 - 344)	216.5 (37 - 603)	$1x10^{-10}$
GHb (%)	8.2 ± 1.9	9.3 ± 2.2	$1x10^{-3}$
rs11721827 (A/C)	**A/A**	**A/C + C/C**	
Gender (% male)	57.6	55.0	0.821
Age (years)	40.5 ± 11.1	37.1 ± 14.1	0.020
Age at diagnosis (years)	17 (1 - 48)	10 (1 - 35)	0.006
FPG (mg/dl)	110 (41 - 603)	217 (37 - 477)	$1x10^{-10}$
GHb (%)	8.7 ± 2.1	9.2 ± 2.2	0.191
rs3775291 (A/G)	**A/A**	**A/G + G/G**	
Gender (% male)	46.3	57.2	0.192
Age (years)	38.9 ± 12.4	38.9 ± 12.3	0.991
Age at diagnosis (years)	8 (2 - 35)	16 (1 - 51)	0.045
FPG (mg/dl)	171 (65 - 349)	155 (37 - 603)	0.425
GHb (%)	8.6 ± 1.9	8.5 ± 1.9	0.998
rs13126816 (A/G)	**A/A**	**A/G + G/G**	
Gender (% male)	47.5	58.4	0.132
Age (years)	39.6 ± 10.2	38.6 ± 12.4	0.675

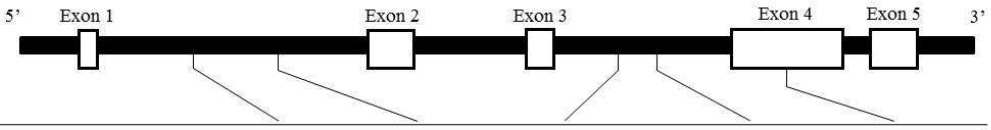
Age at diagnosis	15 (2 - 43)	15 (1 - 51)	0.739
FPG (mg/dl)	132.5 (40 - 422)	165 (37 - 603)	0.816
GHb (%)	9.4 ± 2.4	8.6 ± 2.1	0.073
rs7668666 (A/C)	**A/A**	**A/C + C/C**	
Gender (% male)	59.2	55.6	0.665
Age (years)	37.2 ± 12.6	38.5 ± 12.5	0.532
Age at diagnosis (years)	17 (2 - 41)	15 (1 - 51)	0.928
FPG (mg/dl)	147 (64 - 324)	155 (37 - 603)	0.680
GHb (%)	8.1 ± 1.5	8.7 ± 2.1	0.180

FPG: fasting plasma glucose; GHb: glycated hemoglobin. *P values were obtained from Student's t-test or χ^2 test. Only P values lower than the Bonferroni threshold (0.01) were considered statistically significant.

Table 4. Haplotypes of the *TLR3* gene in T1DM patients and non-diabetic subjects

Haplotypes	T1DM patients* (n = 956)	Non-diabetic subjects* (n = 828)	Frequency in the total sample
Ht1 [A G C A A]	0.035	0.027	0.033
Ht2 [A G C A G]	0.130	0.132	0.131
Ht3 [A G C C A]†	0.009	0.058	0.032
Ht4 [A G C C G]†	0.248	0.275	0.261
Ht6 [A G T A G]	0.016	0.023	0.020
Ht8 [A G T C G]	0.055	0.074	0.066
Ht9 [A A C A A]†	0.042	0.017	0.032
Ht10 [A A C A G]†	0.022	0.008	0.017
Ht11 [A A C C A]	0.128	0.129	0.133
Ht12 [A A C C G]†	0.098	0.071	0.089
Ht15 [A A T C A]	0.010	0.008	0.009
Ht16 [A A T C G]†	0.029	0.015	0.024
Ht20 [C G C C G]	0.021	0.026	0.024
Ht21 [C G T A A]	0.010	0.007	0.009
Ht22 [C G T A G]	0.014	0.016	0.015
Ht24 [C G T C G]	0.060	0.042	0.050
Ht27 [C A C C A]	0.012	0.011	0.011

n = number of chromosomes. Data are presented as proportion. The first letter of the haplotypes refers to the rs11721827 polymorphism, the second to the rs13126816 polymorphism, the third to the rs5743313 polymorphism, the forth to the rs7668666 polymorphism and the fifth to the rs3775291 polymorphism. * Permutation P value = 0.001 for comparisons of haplotype frequencies between groups. † Adjusted residuals which deviated from expected values (P values < 0.05).

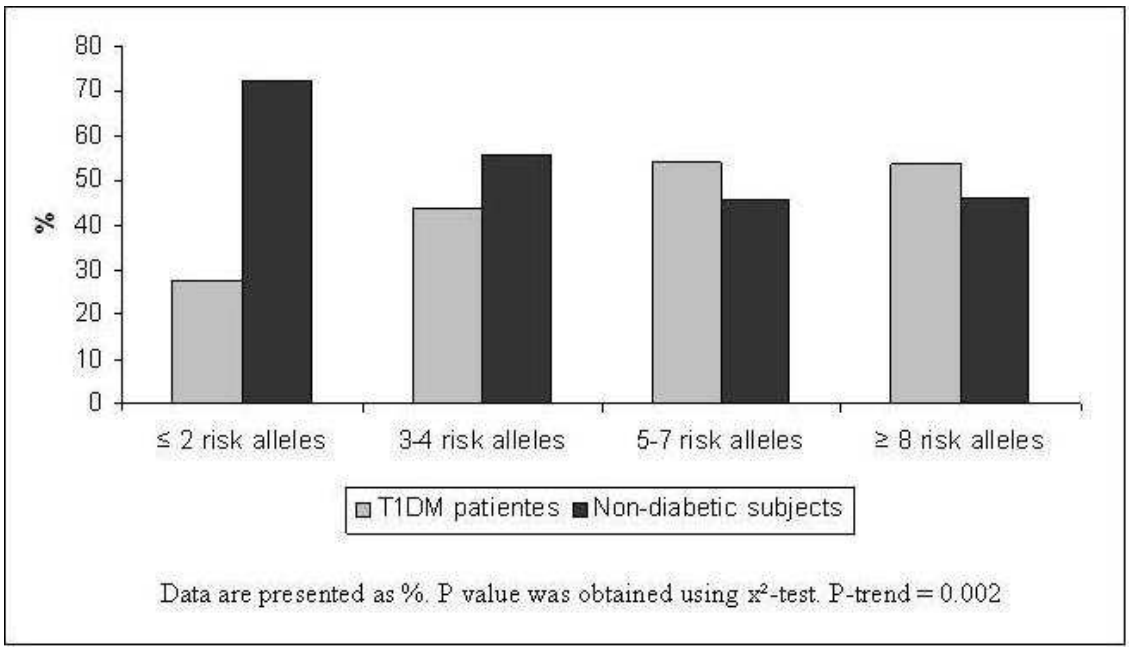


Genome position	dsSNP ID	rs11721827	rs13126816	rs5743313	rs7668666	rs3775291
186991137	**rs11721827**		0.159	0.540	0.138	0.270
186994178	**rs13126816**	0.011		0.311	0.081	0.696
187000367	**rs5743313**	0.233	0.013		0.467	0.856
187001292	**rs7668666**	0.109	0.001	0.229		0.155
187004074	**rs3775291**	0.007	0.434	0.089	0.004	

|D'| (right) **r^2** (left)

Figure 1. Genomic structure of *TLR3* gene and pairwise linkage disequilibrium (LD) values for the five analyzed polymorphisms in this gene. In the upper part of the figure, the *TLR3* gene structure and the positions of the analyzed polymorphisms are indicated. In the lower part, simple pairwise LD values, |D'| (right) and r^2 (left), are shown.

Figure 2. T1DM patients and non-diabetic subjects broken down by the number of risk alleles of the analyzed polymorphisms in the estimated *TLR3* haplotypes.



Data are presented as %. P value was obtained using x²-test. P-trend = 0.002

CONCLUSÕES GERAIS

Os resultados do presente estudo indicam que os polimorfismos rs3775291 (A/G) e rs13126816 (A/G) no gene *TLR3* estão associados com risco para o DM1 em indivíduos do Sul do Brasil. Além disso, a prevalência da doença aumenta conforme aumenta o número de alelos de risco (mutados) dos cinco polimorfismos estudados nos haplótipos formados, indicando que o gene *TLR3* pode interferir na suscetibilidade ao DM1. Os polimorfismos rs11721827 (A/C) e rs5743313 (C/T) no gene *TLR3* parecem estar associados à idade precoce de diagnóstico da doença e a um pior controle glicêmico na nossa população.

Embora mais estudos sejam necessários para avaliar tanto o efeito do gene *TLR3* quanto do polimorfismo rs3775291 (A/G; Leu412Phe) na patogênese do DM1, hipotetizamos que durante infecções virais com tropismo pelas células-beta, uma atividade atenuada de TLR3, como parece ocorrer na presença do alelo 412Phe do polimorfismo rs3775291, pode levar a uma resposta de IFN-I reduzida, uma replicação viral incontrolada e, consequentemente, ao DM1.