UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS MÉDICAS:

ENDOCRINOLOGIA

ESTUDO DA ASSOCIAÇÃO DO POLIMORFISMO rs1990760 (G/A) NO GENE *IFIH1* COM DIABETES MELLITUS TIPO 1 E COM A EXPRESSÃO DESTE GENE EM CÉLULAS MONONUCLEARES HUMANAS

DISSERTAÇÃO DE MESTRADO

ANA PAULA BOUÇAS

Porto Alegre, fevereiro de 2013

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"Foi o tempo que dedicaste à tua rosa que a fez tão importante"

Antoine de Saint-Exupéry

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- Artigo de revisão: "The role of the interferon induced with helicase c domain 1 (IFIH1) on the development of type 1 diabetes mellitus" (a ser submetido aos ABEM)

- Artigo original: "Arterial hypertension in patients with type 1 diabetes mellitus is associated with the A allele of the rs1990760 (G/A) polymorphism in the *IFIH1* gene and with expression of this gene in human mononuclear cells" (submetido à revista Diabetologia).

SUMÁRIO

LISTA DE ABREVIATURAS

3′-UTR	3′ untranslated region
AP-1	Activator protein 1
ATP	Adenosine triphosphate
BP	Blood pressure
CARD	Caspase activation and recruitment domains
CCL2	Chemokine (C-C motif) ligand 2
CCL5	Chemokine (C-C motif) ligand 5
CTLA4	Cytotoxic T-lymphocyte associated protein 4
CV-B	Coxsackievirus B
CXCL10	C-X-C motif chemokine 10
dsRNA	Double-stranded RNA
GAD65	Glutamic acid decarboxylase
GWAS	Genome-wide association studies
HLA	Human leukocyte antigen
HNF-3	Hepatocyte nuclear factor 3-alpha
HO-1	Heme oxygenase-1
HWE	Hardy–Weinberg equilibrium
IA-2	(insulinoma antigen 2 antibodies)
IA-2β	Tyrosine phosphatases
IFIH1	Interferon induced with helicase C domain 1
IFN-γ	Gamma- interferon
IFN-α	Alpha-Interferon

IFN-1	Type 1 interferon
IFNs	Interferons
IKK-β	inhibitor of nuclear factor kappa-B kinase subunit beta
IKK-α	Inhibitor of nuclear factor kappa-B kinase subunit alpha
IL-1β	Interleukin-1 beta
IL-15	Interleukin 15
IL2RA	Interleukin 2 receptor – alpha
INS-1E	Rat insulinoma cell line
IPS-1	IFN-β promoter stimulator-1
IRF-3	IFN-regulatory factor 3
JAK	Janus kinase
LGP2	Laboratory of Genetics and Physiology-2
MDA-5	Melanoma differentiation-associated gene-5
MHC	Major histocompatibility complex
MPH1	Mutator phenotype protein 1
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-kappa B
NLRs	NOD-like receptors
NOD	Nucleotide-binding oligomerization domain
NOD mice	Non-obese diabetic mice
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PIC	Polyinosinic-polycytidylic acid
PRRs	Pattern-recognition receptors

PTPN22	Protein tyrosine phosphatase nonreceptor type 22
RIG-I	Retinoic acid-inducible gene I
RLHs	RIG-I-like helicases
SAH	Systemic arterial hypertension
siRNA	Small interfering RNA
SNPs	single-nucleotide polymorphism
ssRNA	Single-stranded RNA
STAT	Signal Transducer and Activator of Transcription
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TBK-1	Serine/threonine-protein kinase
TLR3	Toll-like receptor 3
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
ZnT8	Zinc transporter

RESUMO

Introdução e objetivos: O diabetes mellitus tipo 1 (DM1) corresponde de 5-10% dos casos de diabetes e resulta da destruição autoimune das células-beta pancreáticas, tornando os indivíduos afetados dependentes de insulina para a sobrevivência. A autoimunidade contra as células-beta é desencadeada por fatores ambientais atuando em combinação com uma predisposição genética. Agentes virais parecem ser um dos desencadeadores ambientais do DM1. O gene interferon induced with helicase C domain 1 (*IFIH1)* tem um importante papel na defesa imunológica contra viroses, uma vez que desencadeia a liberação de citocinas pelas células do sistema imune induzindo apoptose das células infectadas por vírus. Por isso, ele é um gene candidato ao desenvolvimento do DM1. De fato, o polimorfismo rs1990760 (G/A) no gene *IFIH1* tem sido associado com o desenvolvimento do DM1 em algumas populações. No presente estudo, nós investigamos se o polimorfismo rs1990760 (G/A) no gene *IFIH1* está associado ao DM1 ou as suas características clínicas e laboratoriais. Além disso, analisamos a expressão deste gene em células mononucleares humanas de pacientes com DM1.

Métodos: No estudo de associação, as frequências do polimorfismo rs1990760 (G/A) no gene *IFIH1* foram avaliadas em 527 pacientes com DM1 e em 517 indivíduos não-diabéticos. A expressão do gene *IFIH1* foi analisada por RT-qPCR em células mononucleares coletadas de um subgrupo de 26 pacientes com DM1.

Resultados: As frequências alélicas e genotípicas do polimorfismo rs1990760 (G/A) não diferiram estatisticamente entre pacientes com DM1 e indivíduos não-diabéticos ($p = 0,139$ e $p = 0,129$; respectivamente). Interessantemente, pacientes com DM1 portadores do alelo A (A/A ou G/A) apresentaram menores níveis de pressão arterial

sistólica (p = 0,001) e diastólica (p < 0,000001) quando comparados com pacientes com o genótipo G/G. Além disso, o genótipo A/A foi associado com proteção para hipertensão arterial sistêmica (HAS) após ajuste para covariáveis (RC = 0,339, p = 0,019). O polimorfismo rs1990760 (G/A) não foi associado com níveis pressóricos ou presença de HAS em 725 pacientes com diabetes mellitus tipo 2 da mesma população. Os níveis de expressão do gene *IFIH1* em células mononucleares de pacientes com DM1 não diferiram significativamente entre os diferentes genótipos do polimorfismo rs1990760 (G/A). Entretanto, os níveis de expressão do gene *IFIH1* se mostraram aumentados em pacientes com DM1 com HAS quando comparados a pacientes com DM1 normotensos [6.7 (1.7-41.2) *vs*. 1.8 (1.3-73.3) unidades arbitrárias, respectivamente; p = 0.036].

Conclusões: A presença do alelo A do polimorfismo rs1990760 (G/A) no gene *IFIH1* parece estar associada com proteção para HAS em pacientes com DM1. Este é o primeiro estudo que demonstra a associação deste polimorfismo com HAS, sendo necessária a realização de estudos adicionais para avaliar o papel funcional deste gene na patogênese da hipertensão.

ABSTRACT

Introduction/aims: Type 1 diabetes mellitus (T1DM), which accounts for 5-10% of those with diabetes, results from a cellular-mediated autoimmune destruction of the pancreatic beta-cells, which renders patients insulin-dependent for life. The triggering of autoimmunity against beta-cells is caused by environmental factors acting in combination with a predisposition genetic background. Viral pathogens seem to be one of the environmental triggers of T1DM. The *IFIH1* gene has an important role in the immune defense against viruses since it triggers the production of cytokines by immune system cells; thus, inducing apoptosis of virus-infected cells. Hence, *IFIH1* is a candidate gene for development of T1DM. In fact, the rs1990760 (G/A) polymorphism in the *IFIH1* gene has been associated with the development of T1DM in some populations. In the present study, we investigated whether the rs1990760 (G/A) polymorphism in the *IFIH1* gene is associated with T1DM or its clinical and laboratory characteristics. In addition, we analyzed the expression of this gene in human mononuclear cells from T1DM patients.

Methods: In the association study, the frequencies of the *IFIH1* rs1990760 (G/A) polymorphism were evaluated in 527 patients with T1DM and in 517 non-diabetic subjects. *IFIH1* gene expression was analyzed by RT-qPCR in mononuclear cells collected from a subgroup of 26 T1DM patients.

Results: Allele and genotype frequencies of the rs1990760 (G/A) polymorphism did not differ significantly between T1DM patients and non-diabetic subjects ($P = 0.139$ and $P = 0.129$, respectively). Interestingly, T1DM patients carrying the A allele (A/A or G/A) showed lower levels of systolic ($P = 0.001$) and diastolic ($P < 0.000001$) blood

pressures as compared to patients with the G/G genotype. Moreover, the A/A genotype was associated with protection for arterial hypertension (AH) after adjustment for covariates (OR = 0.339, P = 0.019). The rs1990760 (G/A) polymorphism was not associated with blood pressure levels or presence of AH in 725 patients with type 2 diabetes mellitus from the same population. *IFIH1* gene expression in mononuclear cells from T1DM patients did not differ significantly among the different genotypes of the rs1990760 (G/A) polymorphism. However, *IFIH1* gene expression was increased in T1DM patients with AH as compared to normotensive T1DM patients [6.7 (1.7-41.2) *vs*. 1.8 (1.3-73.3) arbitrary units, respectively; P = 0.036].

Conclusions: The presence of *IFIH1* rs1990760 A allele seems to be associated with protection for AH in T1DM patients. This is the first study reporting an association between this polymorphism and hypertension. Further studies are needed to assess the functional role of this gene in the pathogenesis of hypertension.

Parte I – Artigo de revisão

THE ROLE OF INTERFERON INDUCED WITH HELICASE C DOMAIN 1 (IFIH1) IN THE DEVELOPMENT OF TYPE 1 DIABETES MELLITUS

THE ROLE OF INTERFERON INDUCED WITH HELICASE C DOMAIN 1 (IFIH1) IN THE DEVELOPMENT OF TYPE 1 DIABETES MELLITUS

O PAPEL DO INTERFERON INDUZIDO COM O DOMÍNIO C DA HELICASE I (IFIH1) NO DESENVOLVIMENTO DO DIABETES MELLITUS TIPO 1

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Short title: Role of IFIH1 in the development of type 1 diabetes.

SUMMARY

Type 1 diabetes mellitus (T1DM) is a chronic, progressive autoimmune disease characterized by metabolic decompensation frequently leading to dehydration and ketoacidosis. Viral pathogens seem to play a major role in triggering the autoimmune destruction that leads to the development of T1DM. Among several viral strains investigated so far, the enteroviruses have been consistently associated with 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 *IFIH1* gene codes for a cytoplasmic receptor of the pattern-recognition receptors (PRRs) family that recognizes dsRNA, playing a role in the innate immune response triggered by viral infection. Binding of dsRNA to this PRR triggers the release of proinflammatory cytokines, such as interferons (IFNs), which exhibit potent antiviral activity, protecting uninfected cells and inducing apoptosis of infected cells. The *IFIH1* gene appears to play a major role in the development of some autoimmune diseases, and is therefore a candidate gene for T1DM.

Keywords: Autoimmunity, type 1 diabetes mellitus, viral infection, IFIH1

RESUMO

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 até agora, a família dos enterovírus foi consistentemente 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 de RNA e DNA viral. O gene *IFIH1* codifica um receptor citoplasmático pertencente à família dos *pattern-recognition receptors* (PRRs) que reconhece o RNAfd, tendo um papel importante na resposta imune inata desencadeada por infecção viral. A ligação do RNAfd a esta PRR desencadeia a liberação de citocinas proinflamatórias como interferons (IFNs), os quais exibem uma potente ação antiviral e têm como objetivo proteger as células não infectadas e induzir apoptose naquelas já contaminadas. O gene *IFIH1* parece ter uma participação importante no desenvolvimento de algumas doenças autoimunes. Por isso, este gene é um candidato ao desenvolvimento do DM1.

Palavras-chaves: autoimunidade, diabetes mellitus tipo 1, infecção viral, IFIH1

INTRODUCTION

Type 1 diabetes mellitus (T1DM), which accounts for 5–10% of all cases of diabetes mellitus, is characterized by severe insulin deficiency secondary to autoimmune destruction of pancreatic beta-cells. Consequently, subjects with T1DM are usually dependent on insulin injections for life [1-4]. Markers of autoimmune beta-cell destruction include autoantibodies to insulin, to the islets of Langerhans, to glutamic acid decarboxylase (GAD65), to the beta-cell-specific zinc transporter ZnT8, and to the tyrosine phosphatases IA-2 and IA-2β [4]. One—but usually more—of these antibodies are present in 85–90% of patients when fasting hyperglycemia is initially detected [4].

Inflammation of the islets of Langerhans (insulitis) probably develops within the context of a "dialog" between immune cells and beta-cells. This dialog is mediated partly by cytokines and chemokines, which are released both by immune cells and by beta-cells themselves, 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 instead to the resolution of insulitis [5]. 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 variety of environmental triggers [2, 6, 7]. Among the various loci associated with T1DM, the human leukocyte antigen (*HLA*) class II locus on chromosome 6p21 is, by far, the leading genetic risk factor for T1DM, accounting for 30–50% of genetic risk for the condition [8]. Other genes are associated with relatively minor effects on T1DM risk as 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, the interleukin 2 receptor – alpha (*IL2RA*) gene, the interferon induced with helicase C domain 1 (*IFIH1*)

gene and other genes recently discovered by means of genome-wide association studies (GWAS) (**Figure 1**) [8, 9].

The environmental triggers and potentiators of autoimmune beta-cell destruction include viral infections, dietary exposures during childhood (e.g. to cow's milk), vaccination, and toxins [2, 10, 11]. There is substantial evidence that viral pathogens, such as enteroviruses, rubella virus, mumps virus, rotaviruses, parvoviruses, and cytomegalovirus, play a major role in triggering the autoimmune destruction of pancreatic beta-cells [12, 13]. Among these viral strains, particular attention is given to the enteroviruses, which exhibit specific tropism for the pancreas and have been associated with the development of T1DM in humans [2, 14, 15]. 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 [2, 11]. Furthermore, coxsackieviruses isolated from patients with T1DM were able to induce diabetes in susceptible mice [16]. In a recent study, the Coxsackie B4 virus was identified in 50% of samples collected from patients with T1DM and was also able to infect human islets *in vitro*, impairing insulin secretion in response to glucose [17].

Microbial recognition by the mammalian immune system relies on components of both innate and adaptive immunity. Innate immunity is the first line of defense against bacteria, fungi, and viruses. Detection of 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 viral RNA replication and transcription [18-20]. Innate immune system cells, such as macrophages and dendritic cells, kill invading microorganisms through phagocytosis or induction of cytokine

production. Furthermore, innate immunity activates the 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 [19].

Some studies have shown that certain PRRs, such as *IFIH1*, play a role in the development of T1DM in animal models [5, 21, 22]. The *IFIH1* gene, also known as the melanoma differentiation-associated gene-5 (*MDA-5*), codes for a cytoplasmic receptor that recognizes dsRNA and is involved in the innate immune response triggered by viral infection [23]. Binding of dsRNA to IFIH1 triggers the release of proinflammatory cytokines, particularly interferons (IFNs), by immune cells, thus inducing apoptosis of virus-infected cells [18, 24]. Therefore, *IFIH1* is a candidate gene for T1DM susceptibility. Within this context, the objective of the present review was to address the role of *IFIH1* in the development of T1DM.

THE ROLE OF PATTERN RECOGNITION RECEPTORS (PRRs) IN THE RESPONSE TO VIRAL INFECTION

As mentioned above, recognition of pathogens by the innate immune system relies on PRRs, which constitute the first line of defense against microbial infection [25, 26]. Recent studies have identified three groups of PRRs: toll-like receptors, retinoic acid-inducible gene I (RIG-I)-like helicases (RLHs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [27, 28]. The RLH class comprises the helicases IFIH1/MDA-5, retinoic acid-inducible gene 1 (*RIG-I*), and Laboratory of Genetics and Physiology-2 (*LGP2*).

During viral infection, dsRNA or single-stranded RNA (ssRNA) are recognized by specific PRRs, present on infected cells, that undergo conformational changes and activate signaling cascades that ultimately drive production of several proinflammatory cytokines, chemokines, and type I IFNs (INF-I) [18, 29]. IFN-1 is a cytokine produced by most cells during viral infection and promotes the expression of several genes involved with antiviral response in target cells and also act as a modulator of the adaptive immune system by activating dendritic cells, T lymphocytes, and B lymphocytes [30]. These IFNs exhibit potent antiviral action, protecting uninfected cells and inducing apoptosis of infected ones, which is partially caused by endoplasmic reticulum stress [27]. Interestingly, high levels of IFN-1 are found in the pancreas of patients with T1DM, and IFN-α is known to contribute to the development of experimental viral-induced diabetes [5]. Furthermore, IFN-I treatment appears to protect against the development of T1DM and reduce the incidence of the disease in NOD mice [31].

Human pancreatic islets infected with the Coxsackie B5 virus or exposed to IFN-α or IFN-γ + IL-1β exhibit increased expression of Toll-like receptor 3 (TLR3) and RLHs (RIG-1 and IFIH1) [32, 33]. 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 key transcription factors: nuclear factor-kappa B (NF-κB) and IFN-regulatory factor 3 (IRF-3) [34-36]. Although TLR3 was the first dsRNA sensor identified as being able to activate NF-κB and IRF3, its role as a primary antiviral receptor was recently called into question [37]. *In vivo* antiviral responses against a wide range of viral pathogens, including the vesicular stomatitis virus, reoviruses, murine cytomegalovirus, and lymphocytic choriomeningitis virus, were similar in wild-type and *TLR3* knockout mice [38]. Indeed, more recent

studies show that, whereas NF-κB and IRF3 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-1 and IFIH1 [34, 35]. Activation of NF-κB and IRF-3 triggers production of IFN-α and IFN-β, leading to activation of the Jak/STAT-1 pathway and triggering the expression of MHC class I antigens and a variety of chemokines [26, 32, 35, 39]. 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 [5].

THE INTERFERON INDUCED WITH HELICASE C DOMAIN 1 (IFIH1) RECEPTOR

The IFIH1 protein belongs to a family of helicases that also comprises two other members: the RIG-I and LGP2 receptors [26]. RNA helicases are highly conserved enzymes that use energy derived from ATP hydrolysis to bind dsRNA, destabilizing and unwinding it [40]. IFIH1 and RIG-I contain two CARD (N-terminal caspase activation and recruitment domains) effector domains essential to their signaling activity. Furthermore, all three helicases contain a DExD/H-box-type RNA helicase domain, which is also essential to their function. A C-terminal domain was recently identified as the site of dsRNA binding in all three helicases [26, 41, 42]. The LGP2 helicase does not contain CARD domains and is probably unable to activate downstream signaling

pathways. LGP2 also recognizes dsRNA, but appears to act as a negative regulator, interfering with viral RNA recognition by IFIH1 and RIG-I [42]. Depending on the type of virus, LGP2 may also act as a positive regulator of its other RLH counterparts [19].

After binding virus-derived dsRNA, IFIH1 and RIG-I interact, via their CARD domains, with the IPS-1 adaptor molecule (IFN-β promoter stimulator-1, also known as Cardif, MAVS or VISA), which recruits intermediary signaling molecules, such as IKK-α, -β, -ε, and TBK1, ultimately leading to NF-κB and IRF-3 activation [20, 26, 41, 42]. Furthermore, TRAF3, an antiviral molecule implicated in the production of type I IFN, also plays a role in these intracellular signaling events [37]. As described elsewhere, activation of NF-κB and IRF-3 induces expression of IFN-β and several genes regulated by these transcription factors, which will direct inflammatory and antiviral responses against infection (**Figure 2**) [26, 42].

IFIH1 and RIG-I share a sequence homology of 25% within the CARD domains and 40% within the helicase domain [41]. Experimental data suggest that these two helicases use similar intracellular signaling mechanisms to induce an antiviral response. However, despite their structural similarities, IFIH1 and RIG-I appear to be non-redundant, and are involved in the recognition of different types of dsRNA and ssRNA virus. Whereas RIG-I recognizes the hepatitis C virus, several paramyxoviruses (mumps virus, varicella zoster virus, respiratory syncytial virus, parainfluenza virus), the vesicular stomatitis virus and the influenza A virus, IFIH1 recognizes *Picornaviridae*, such as rhinoviruses, echoviruses, enteroviruses, and the encephalomyocarditis virus [23, 43]. Furthermore, *IFIH1* knockout mice ($IFIH1^{-/-}$) fail to produce IFN-α in response to exposure to the synthetic dsRNA polyinosinic:polycytidylic acid (PIC), which demonstrates that IFIH1 is the primary sensor for PIC [23]. Interestingly, these two helicases appear to recognize reoviruses, the West Nile virus, and the dengue virus

[26]. Experiments involving PIC infection have shown that short dsRNA segments (< 2000bp) activate RIG-I, whereas long dsRNA segments (> 2000bp) are best recognized by IFIH1 [44, 45]. Nevertheless, the mechanisms responsible for distinguishing long dsRNA segments from short ones have yet to be elucidated.

The *IFIH1* gene was identified by subtractive hybridization as a novel gene overexpressed in HO-1 human melanoma cells induced to differentiate by treatment with IFN-β and mezerein, a protein kinase C activator [46]. The *IFIH1* gene is located on chromosome 2 (region 2q24.3), contains 16 exons (**Figure 3**) and codes for a 1,025-amino acid protein with a molecular mass of 116.7 KDa [42, 47]. This helicase is expressed at low levels in a wide range of tissues, including pancreatic beta-cells, but is expressed at relatively high concentrations in immune cells [33, 47]. At the transcriptional level, IFIH1 expression is induced by IFN-1, retinoic acid, and dsRNA [42].

Huhn *et al*. [48] found that *IFIH1*$^{-/-}$ knockout mice exhibit increased susceptibility to Coxsackie B3 virus infection. Loss of *IFIH1* enabled faster viral replication, leading to hepatomegaly, pancreatic injury, and high mortality rates in these animals. The authors also found that IFIH1 is not required for induction of IFN-1, but is essential for production of peak IFN-α levels after infection. Furthermore, both IFIH1 and TLR3 appear to play a major role in the prevention of diabetes in C57BL/6 mice infected with encephalomyocarditis virus strain D, a beta-cell-tropic virus [49]. Deletion of only one allele of the *IFIH1* gene was enough to cause transient hyperglycemia in mice infected with the virus, whereas mice in which both copies of the gene were deleted exhibited severe cardiac pathology [49]. Colli *et al*. [30] assessed the effect of IFIH1 blockade by transfecting primary beta-cells and INS-1E beta-cells (a rat insulinoma cell line) with anti-IFIH1 siRNA and exposing these transfected cells to

PIC. As expected, PIC increased *IFIH1* gene expression in these cells, which was inhibited by siRNA. Inhibition of IFIH1 in primary and INS-1E beta-cells did not inhibit PIC-induced apoptosis; however, it did reduce expression of proinflammatory cytokines (IFN-β and IL-15) and chemokines (CCL2, CCL5 and CXCL10), suggesting that IFIH1 is not essential for PIC-induced beta-cell death, but rather regulates important inflammatory signaling mechanisms in these cells [30].

In mice, increased gene expression of *IFIH1* leads to a state of chronic IFN-1 production, characterized by resistance to lethal viral infections [50]. Hultcrantz *et al*. [33] showed that, in human pancreatic islets, IFN-induced antiviral defenses provide a powerful protective mechanism against replication of coxsackieviruses. Treatment with IFN-α is known to increase gene expression of the chemokine CXCL10 in human islets [33]. This chemokine, when produced during infection, leads to T-cell recruitment to the islet site and appears to play a key role in host defense against islet-tropic viruses in individuals susceptible to T1DM [33].

These studies further strengthen the hypothesis that *IFIH1* plays a key role in the regulation of local islet inflammation during viral infection.

IFIH1 gene polymorphisms and their association with T1DM

The association between *IFIH1* and T1DM was first reported in 2006, by Smyth *et al*. [51], who conducted GWAS in European families affected by T1DM within a large Caucasian population cohort from the United Kingdom, for a total of over 10,000 subjects. Several *IFIH1* polymorphisms were associated with T1DM, with the rs1990760 (G/A) polymorphism, which substitutes an alanine to valine in codon 946 of exon 15, being most strongly associated with protection against development of the disease (odds ratio [OR] = 0.86, $P = 1.42 \times 10^{-10}$ for the G allele) [51].

Associations between *IFIH1* polymorphisms and T1DM have been replicated in some populations, [29, 52-56], but not in others [57-59] (**Table 1**). Jermendy *et al*. [53] studied the rs1990760 polymorphism in Hungarians and Finns and conducted a meta-analysis of 5 studies that analyzed this polymorphism in T1DM patients and nondiabetic controls, including their own study. In Hungarians, the A allele of this polymorphism was strongly associated with T1DM (OR = 1.29; P = 0.002). Furthermore, the meta-analysis showed a significant association between the A allele and risk of developing T1DM (OR = 1.18; $P = 5.3 \times 10^{-15}$).

Qu *et al*. [54] assessed three single nucleotide polymorphisms (SNPs) located in the *IFIH1* gene or its adjacent intergenic regions (rs1990760, rs3747517 and rs2111485) in 589 French Canadian nuclear family trios. The rs1990760 and rs3747517 polymorphisms showed a trend toward association with T1DM as reported by other studies, but the effect did not reach statistical significance, most likely due to weak statistical power [54]. Conversely, the A allele of SNP rs2111485 was associated with protection for T1DM (OR = 0.84, $P < 0.05$). Yang *et al*. [56], in a study of Han Chinese subjects, also failed to find an association between the rs1990760 polymorphism and T1DM, but did find an association between the rs3747517 polymorphism and the condition ($P > 0.001$).

A GWAS of Caucasian subjects in the United States (Georgia and Denver populations) showed that two SNPs in the coding region of *IFIH1* (rs1990760 and rs35744605) and two SNPs in the adjacent 3′ intergenic region (rs2111485 and rs13422767) were associated with increased risk of T1DM (OR = 1.7–1.9), but only in the Georgia population, with the lowest P-value obtained for the rs1990760 polymorphism ($P = 8 \times 10^{-8}$) [52]. Interestingly, the G/G genotype of SNP rs1990760 was associated with increased levels of *IFIH1* expression in the peripheral blood

mononuclear cells of 374 subjects (187 patients with T1DM and 187 nondiabetic controls) [52]. The most common homozygous genotypes for the three other polymorphisms of interest were also associated with increased *IFIH1* expression, which suggests that increased expression of this gene may be associated with greater susceptibility to T1DM development [52]. Nejentsev *et al.* [29] reported that four rare variants of the *IFIH1* gene (rs35337543, rs35667974, rs35744605 and rs35732034), as well as the rs1990760 polymorphism, were independently associated with protection for T1DM in a British population (OR = 0.51–0.84; P=1.3 x 10^{-3} to 2.1 x 10^{-16}).

The rs1990760 polymorphism is not located in any functional region of the protein [42], but the G allele is highly conserved among mammals and may have other, yet-unknown functions, or may affect active domains by means of effects on the tertiary structure [51]. However, Shigemoto *et al*. [60] have shown that this variant has no significant effect on dsRNA binding to IFIH1 or on the IFN activation. The rs3747517 polymorphism replaces histidine to arginine in codon 843 of exon 13, within the MPH1 domain of the protein, which is conserved in ERCC4-like helicases and comprises two functional subdomains (helicase and C-terminal). Liu *et al*. [52] suggest that the rs1990760 polymorphism may affect the sequence of the HNF-3b transcription factor binding site in the *IFIH1* gene, whereas the rs3747517 polymorphism may alter the binding site for the AP-1 transcription factor. As both polymorphisms are located 45–50kb from the start codon, it has yet to be determined whether these variants really play a role in regulation of *IFIH1* expression or are merely in linkage disequilibrium with a functional polymorphism in this region [42, 52].

The rs13422767 and rs2111485 polymorphisms are located within the 3′ intergenic region adjacent to the *IFIH1* gene, 23kb and 13kb from the end of the 3′-UTR of the gene respectively. These polymorphisms do not change any known transcription

factor binding sites and it is not known whether they contribute to the regulation of *IFIH1* gene expression [52]. Rare variants, however, are predicted to have significant biological effects on the *IFIH1* gene, whether by resulting in a truncated protein product through generation of a stop codon in exon 10 (rs35744605), affecting splicing positions (rs35337543 and rs35732034, position +1 of introns 8 and 14 respectively) or changing a highly conserved amino acid (rs35667974 in exon 14) [29].

Bonifacio *et al*. [61] investigated potential associations between cesarean delivery, islet autoimmunity, and genes involved in T1DM susceptibility in 1650 German children born of one parent with T1DM and followed from birth to onset of anti-islet autoantibodies or diagnosis of T1DM (BABYDIAB study). Children delivered via cesarean section had a twofold higher risk of T1DM as compared with children delivered vaginally (hazard ratio = 2.5; P = 0.001). Furthermore, cesarean delivery was associated with faster progression to T1DM after onset of autoimmunity (P = 0.015). Interestingly, an increased risk of T1DM was only observed in children who were born by cesarean section and were homozygous for the G allele of *IFIH1* rs2111485 polymorphism (12-year risk of developing T1DM = 9.1 in G/G children delivered via cesarean section *vs*. 3% in G/G children delivered vaginally; P = 0.0001).

In short, the above-cited studies suggest that more than one *IFIH1* polymorphism contributes to T1DM susceptibility in several populations. Additional research is required to ascertain the biological effects of these polymorphisms.

Associations between *IFIH1* and other autoimmune conditions

Autoimmune diseases are distinct clinical syndromes characterized by changes in normal immune responsiveness due to a loss of tolerance to one or more host constituents [62]. Furthermore, it is widely known that genetic factors play a substantial

role in the pathogenesis of autoimmune conditions. Accordingly, studies of several loci related to the immune system, such as the *IFIH1* gene, have been conducted in an attempt to provide a better understanding of the pathogenesis of these diseases [63].

Interestingly, some studies have shown strong associations between the rs1990760 polymorphism of the *IFIH1* gene and other autoimmune diseases, such as psoriasis [64, 65], chronic periodontitis [64], polymyositis [66], multiple sclerosis [58, 67], systemic lupus erythematosus [68], and Graves' disease [69].

These associations between *IFIH1* and autoimmune conditions unrelated to viral infection, such as Graves' disease, suggest that IFIH1 may also play an endogenous immunoregulatory role unrelated to its function as a viral receptor [69]. Additional studies are needed to identify the role of *IFIH1* in the pathogenesis of these conditions.

CONCLUSIONS

The *IFIH1* gene plays a major role in the innate immune response triggered by viral infection. Binding of viral replication-derived dsRNA to IFIH1 triggers 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, *IFIH1* is an excellent candidate gene for T1DM. Indeed, several studies conducted in different populations suggest that more than one *IFIH1* polymorphism is associated with T1DM. The rs1990760 polymorphism has also been associated with other autoimmune conditions, such as Graves' disease and systemic lupus

erythematosus. Additional studies are required to elucidate the molecular mechanisms underlying the association between these polymorphisms and T1DM and other autoimmune diseases. 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 T1DM.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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Table 1. Studies of polymorphisms in the *IFIH1* gene and type 1 diabetes mellitus (DM1)

Polymorphism	Population and Design	Results	Reference
rs1990760	United Kingdom (4353 cases and 5842 controls)	Association between the G allele and DM1 (OR = 0.86; P = <0.001).	[51]
rs1990760	Canada (7721 DM1 patients and 9679 controls + 2214 nuclear family trios)	Association with type 1 diabetes risk loci (P = $4.1x10^{-5}$).	[55]
rs1990760	757/509 Hungarian/Finnish childhood-onset DM1 cases; 499/250 Hungarian/Finnish controls; and 529/924 Hungarian/Finnish nuclear family trios	In the Hungarian dataset, the A allele was more frequent among cases than among controls (OR= 1.29; P = 0.002). No association was observed in the Finnish dataset. The A allele was significantly overtransmitted in both family trio datasets.	[53]
rs1990760 / rs2111485	Spain (311 DM1 patients and 535 non-diabetic controls)	No significant association with DM1. The rs1990760 polymorphism showed only a trend towards association with DM1 (OR = 0.85, P = 0.07, for the G allele).	[58]

rs1990760	Belgium (1981 DM1 patients, 2092 non-diabetic controls and 430 case-parent trios)	No significant association with DM1.	[57]
rs1990760 / rs35744605 / rs2111485 / rs13422767	U.S. (Georgia population: 1434 DM1 patients and 1865 non-diabetic controls; Denver population: 612 DM1 patients and 552 controls)	Association of the major alleles of the four polymorphisms with DM1 (OR= 1.7 – 1.9; P= < 0.001).	[52]
rs1990760 / rs3747517 / rs2111485	589 French-Canadian nuclear family trios	Association between allele A of the rs2111485 polymorphism and DM1 (OR = 0.84, P < 0.05). The rs1990760 and rs3747517 polymorphisms showed only a trend towards association with DM1 (P > 0.05).	[54]
rs35667974 / rs35337543 / rs35732034 / rs35744605 / rs1990760	United Kingdom (7853 cases and 9166 controls)	All five polymorphisms were associated with DM1 (OR 0.51 – 0.74; P = 1.3×10^{-3} – 2.1×10^{-16}).	[29]

rs1990760 / rs3747517	Han Chinese (464 DM1 patients and 465 controls)	Association between the rs3747517 polymorphism and DM1 ($P < 0.001$). No association between the rs1990760 and DM1.	[56]
rs6432714 / rs10930046	Northeast Brazil (196 DM1 patients and 176 healthy controls)	No significant association with DM1.	[59]

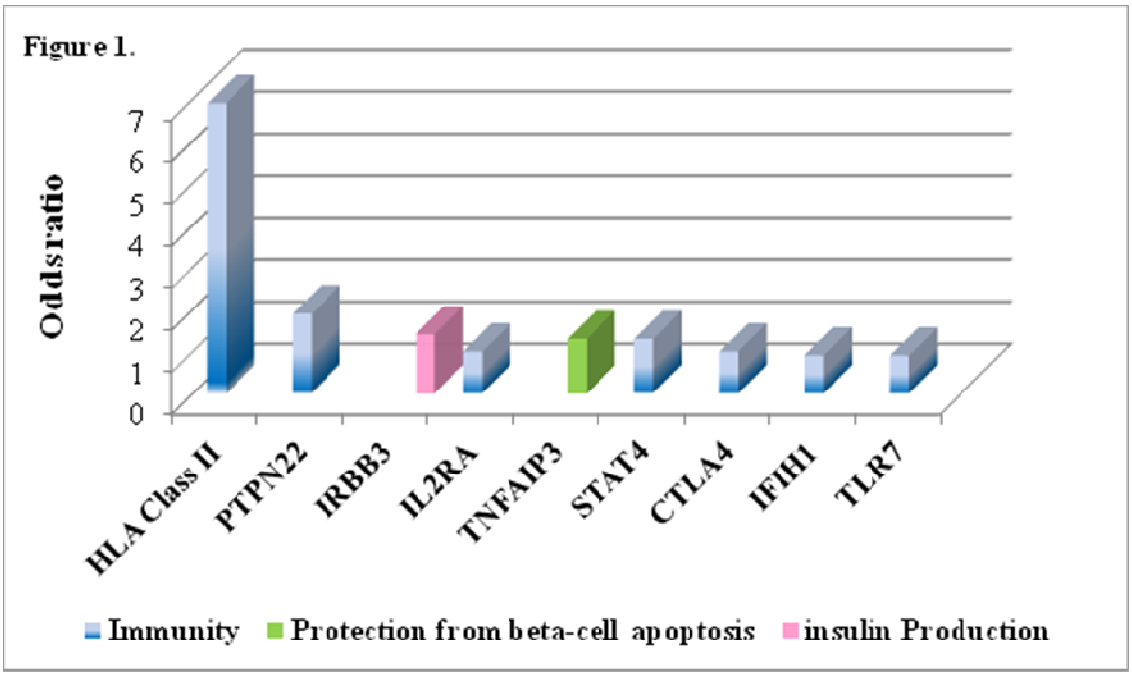

Figure 1. Genes associated with type 1 diabetes mellitus

Odds ratios for susceptibility alleles of nine genes associated with type 1 diabetes mellitus. Figure adapted from Todd *et al*. [9].

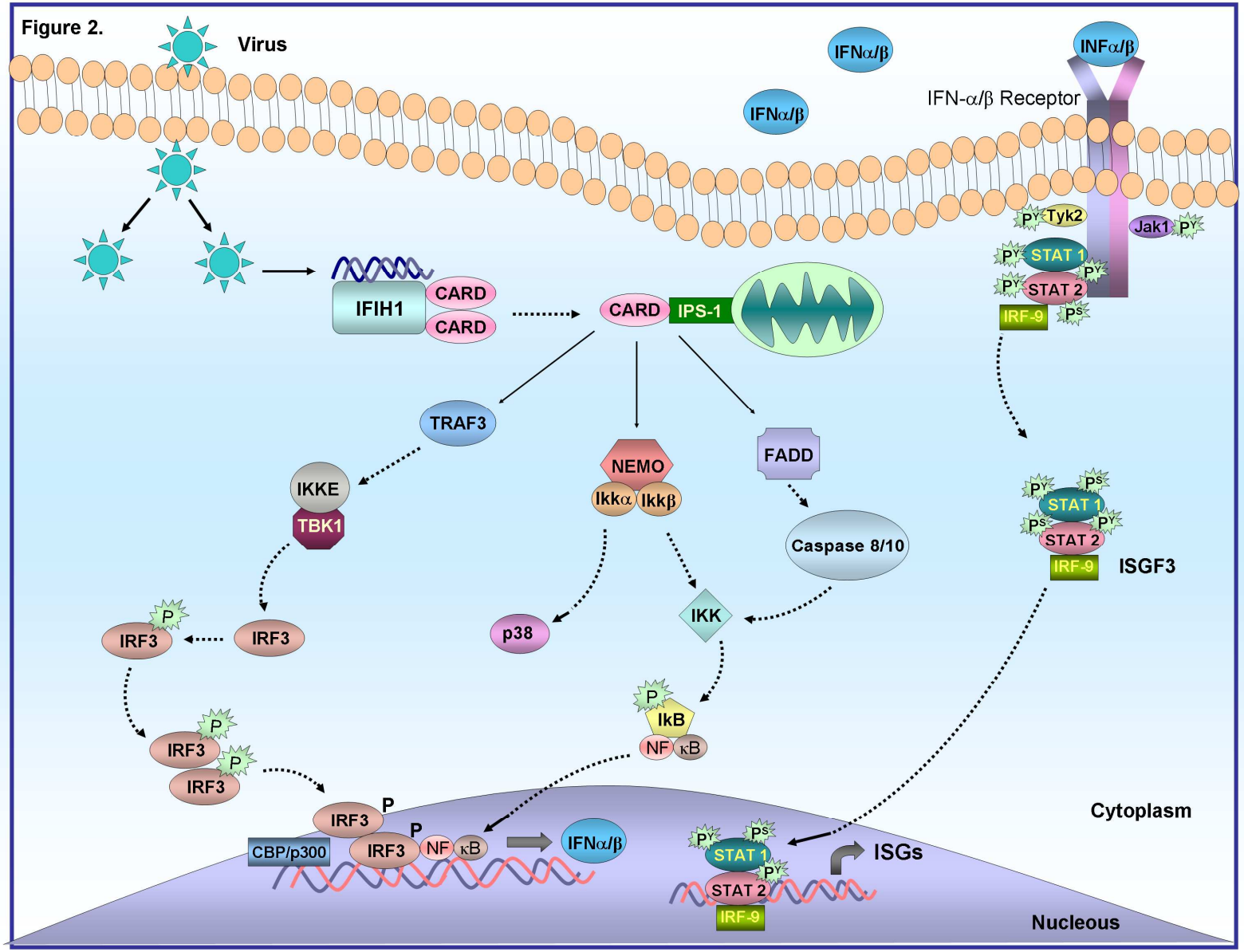
Figure 2.
Virus
IFNα/β
IFNα/β
INFα/β
IFN-α/β Receptor
Tyk2
Jak1
STAT 1
STAT 2
IRF-9
IFIH1
CARD
CARD
CARD
IPS-1
TRAF3
NEMO
Ikkα
Ikkβ
FADD
IKKE
TBK1
Caspase 8/10
ISGF3
IRF3
IRF3
IKK
p38
IkB
NF κB
IRF3
IRF3
IRF3
CBP/p300
IRF3
NF κB
IFNα/β
ISGs
Cytoplasm
Nucleous

Figure 2. Antiviral signaling by IFIH1

Double-stranded RNA (dsRNA) derived from viral replication is detected by the cytoplasmic RNA helicase IFIH1, activating the adaptor protein IPS-1 via CARD domain interactions. IPS-1 then induces intracellular signaling pathways that result in the activation of the transcriptions factors IRF-3 and NF-κB, leading to the production of IFNα/β by infected cells. IFNα/β is then shown signaling through the IFNα/β receptor and the Jak-STAT pathway to drive ISG expression and an innate immune response. See text for further details. Adapted from Wilkins and Gale [26].

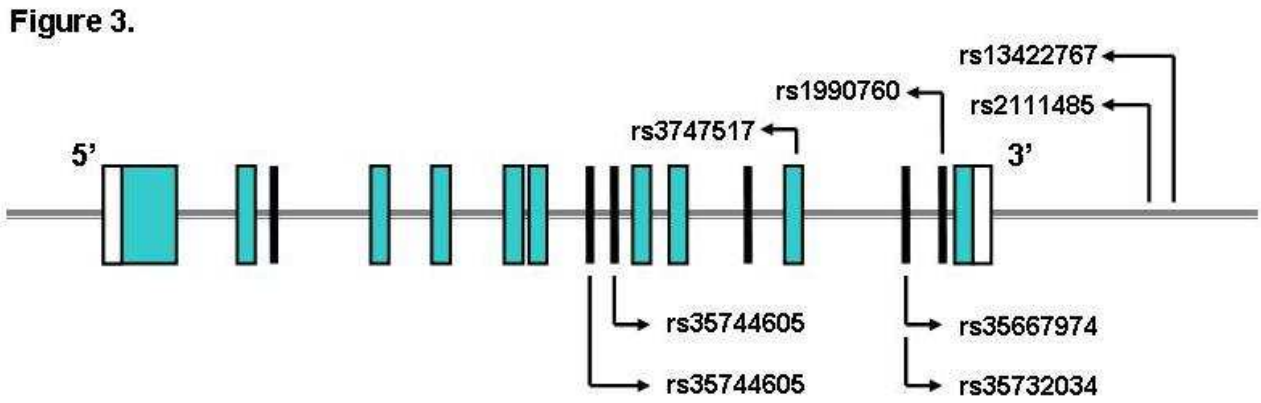

Figure 3. **Map of *IFIH1* locus on chromosome 2 (region 2q24.3)**.

The sixteen exons (boxes) are numbered from left to right according to the transcriptional region. The arrows show the main common polymorphisms associated with type 1 diabetes mellitus. Figure adapted from Chistiakov *et al.* [42].

Parte II – Artigo original

ARTERIAL HYPERTENSION IN PATIENTS WITH TYPE 1 DIABETES MELLITUS IS ASSOCIATED WITH THE A ALLELE OF THE rs1990760 (G/A) POLYMORPHISM IN THE *IFIH1* GENE AND WITH EXPRESSION OF THIS GENE IN HUMAN MONONUCLEAR CELLS

Arterial hypertension in patients with type 1 diabetes mellitus is associated with protection from the A allele of the rs1990760 (G/A) polymorphism in the *IFIH1* gene and with expression of this gene in human mononuclear cells

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Abstract word count: 250

Main text word count: 4123

Abstract

Aims/hypothesis The rs1990760 (G/A) polymorphism of interferon induced with helicase C domain 1 (*IFIH1*) has been proposed to be associated with type 1 diabetes mellitus (T1DM). Here, we investigated whether the rs1990760 polymorphism is associated with T1DM or its clinical and laboratory characteristics in a Southeast Brazilian population, and if *IFIH1* gene expression in mononuclear cells from T1DM patients differs according to the different genotypes of this polymorphism.

Methods Frequencies of the rs1990760 polymorphism were analyzed in 527 T1DM patients and in 517 healthy subjects using TaqMan MGB probes. *IFIH1* gene expressions according to the different genotypes were measured in a sub-sample of 26 T1DM patients by RT-qPCR.

Results Our data confirmed the association of the A allele with risk to T1DM under a dominant model of inheritance (OR=1.421, P=0.037). Surprisingly, T1DM patients carrying the A allele showed lower levels of systolic (P=0.001) and diastolic ($P=1x10^{-10}$) blood pressures as compared to G/G carriers. Furthermore, the A/A genotype was associated with protection to arterial hypertension (AH) after adjustment for covariates (OR=0.339, P=0.019). *IFIH1* gene expression in mononuclear cells from 26 T1DM patients did not differ significantly among the rs1190760 genotypes (P=0.274). Nevertheless, *IFIH1* gene expression was increased in mononuclear cells from T1DM patients with AH as compared with T1DM patients without AH [6.7 (1.7-2.0) *vs*. 1.8 (1.3-7.1) arbitrary units; P=0.036].

Conclusions/interpretations Our results indicate that the rs1990760 polymorphism is associated with T1DM in our population. Interestingly, the rs1990760 A allele seems to be associated with protection for AH in T1DM patients.

Keywords: *IFIH1* gene expression, rs1990760 G/A polymorphism, type 1 diabetes mellitus, arterial hypertension, inflammation.

Abbreviations:

AH Arterial hypertension

AU Arbitrary units

CV-B Coxsackievirus B

DN Diabetic nephropathy

DR Diabetic retinopathy

dsRNA Double-stranded RNA

HWE Hardy–Weinberg equilibrium

IFIH1 Interferon induced with helicase C domain 1

IFN-I Type I interferon

IRF-3 IFN-regulatory factor 3

MDA-5 Melanoma differentiation-associated gene-5

NF-κB Nuclear factor-kappa B

PAMPs Pathogen-associated molecular patterns

PE Preeclampsia

PRRs Pattern-recognition receptors

RIG-I Retinoic acid-inducible gene I

ROS Reactive oxygen species

RT-qPCR RT-quantitative PCR

TLR3 Toll-like receptor 3

T1DM Type 1 diabetes mellitus

T2DM Type 2 diabetes mellitus

Introduction

Type 1 diabetes mellitus (T1DM), which accounts for 5-10% of those with diabetes, results from a cellular-mediated autoimmune destruction of the pancreatic beta-cells, which renders patients insulin-dependent for life [1]. The triggering of autoimmunity against beta-cells is probably caused by environmental factors acting in combination with a predisposing genetic background [2, 3]. The major susceptibility locus maps to the HLA class II genes at chromosome 6p21 and accounts for up to 30-50% of the genetic risk for this disease [1, 4]. Other non-HLA loci have smaller effects on disease risk compared to HLA, and include the *insulin* gene, the *CTLA4* gene, the *PTPN22* gene, the *IL2RA* gene, and the interferon induced with helicase C domain 1 (*IFIH1*) [4].

The hypothesis that viruses may be one of the environmental factors explaining the surprising increase in T1DM incidence during recent decades is strengthened by variations of T1DM incidence from one country to another and from a season to another, as well as by observations relative to the association between immigration and disease development [5]. Epidemiological, experimental and clinical data indicate that the prime viral candidates for causing T1DM in human are enteroviruses, such as Coxsackievirus B (CV-B) [5-7]. CV-B4 is the most common enterovirus found in pre-diabetic and diabetic subjects [7]. One CV-B4 strain isolated from the pancreas of a deceased diabetic child was able to induce diabetes in susceptible mice [8]. Moreover, CV-B4 was identified in the pancreatic tissue from three of six patients with T1DM [9], and it was capable of infecting human islet *in vitro*, impairing glucose-stimulated insulin secretion [10]. The pathogenic role of enteroviruses in T1DM seems to involve damage to beta-cells and local induction of proinflammatory mediators [11].

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 front line of defense against microbial infection [12]. PRRs are evolutionary conserved germ-line-encoded proteins and include Toll-like receptors (TLRs), retinoic acid-inducible I (RIG-I)-like receptors (RIG-I and IFIH1 receptors) and nucleotide-binding oligomerization domain-like receptor (NLR) [13, 14]. These PRRs recognize specific PAMPs in different cellular compartments, such as the plasma membrane, the endossomes or the cytoplasm, and induce the expression of proinflammatory cytokines, chemokines and co-stimulatory molecules which will eliminate the pathogens and activate pathogen-specific adaptive immune responses [14].

The *IFIH1* gene, also known as melanome differentiation-associated gene 5 (*MDA5*), is a functional candidate for T1DM because it encodes a cytosolic receptor that play a major role in the recognition of internal double-stranded RNA (dsRNA), an intermediate nucleic acid generated during the life cycle of most viruses [14, 15], suggesting a potential role in the infectious etiology of T1DM and providing a link between viral infections and this disease. In fact, some studies have demonstrated the association of a non-synonymous polymorphism (rs1990760 G/A [Ala946Thr]) in exon 15 of the *IFIH1* gene with T1DM in more than one population [16-18]. Therefore, the present study investigated whether the *IFIH1* rs1990760 polymorphism is associated with T1DM or its clinical and laboratory characteristics in a Southeast Brazilian population, and if the *IFIH1* gene expression in mononuclear cells from T1DM patients differs according to the different genotypes of this polymorphism.

Subjects and methods

Subjects and phenotype measurements This was a case-control study designed to investigate whether the *IFIH1* rs1990760 polymorphism is associated with T1DM. The diabetic sample comprised 527 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 before the age of 30 years, required insulin for glycemic control within 1 year of diagnosis, and this treatment could not be interrupted thereafter [19]. The non-diabetic sample comprised 517 healthy blood donors who did not have diabetes mellitus or family history for this disease (mean age = 44.0 ± 7.8; male = 55.0%). Additionally, to evaluate whether any association found between clinical characteristics of T1DM and the *IFIH1* rs1990760 polymorphism could be replicated in a non-autoimmune diabetes context, we also analyzed 725 patients with type 2 diabetes mellitus (T2DM) from the same hospital [20]. T2DM was diagnosed according to the ADA guidelines [1]. The ethnic group was defined on the basis of self-classification, and the ethnic proportion was similar between the samples.

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. Assessment of diabetic retinopathy (DR) was performed in all patients by an experienced ophthalmologist using fundoscopy through dilated pupils. DR was classified using the scale developed by the Global Diabetic Retinopathy Group [21]. For the purpose of this study, patients were grouped according to the presence or absence of any degree of DR. Diagnosis of diabetic nephropathy (DN) was based on the albumin excretion rate (AER) in at least two out of three consecutive 24-h timed or random spot sterile urine collections in a 6-month period. Patients were classified as having normoalbuminuria (AER <30 μg/24h or <17 mg/l), microalbuminuria (AER 30-299 μg/24h or 17-173 mg/l) or macroalbuminuria (AER >300 μg/24h or >174 mg/l) [22].

Serum and plasma samples were taken after a 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. Glycated hemoglobin (HbA1c) measurements were performed by different methods and results were traceable to the DCCT method by off-line calibration or through conversion formulae [23]. Total plasma cholesterol, HDL cholesterol and triglycerides were assayed using enzymatic methods. Urinary albumin was measured by immunoturbidimetry (Microalb; Ames-Bayer, Tarrytown NY), and the intra- and interassay coefficients of variation in our laboratory were both <6% [24].

The research protocol was approved by the Hospital´ ethical committee and all subjects gave informed consent in writing.

Genotyping DNA was extracted from peripheral blood leukocytes using a standardized salting-out procedure. The *IFIH1* rs1990760 (G/A) polymorphism was genotyped using

primers and probes contained in the Human Custom TaqMan Genotyping Assay 20x (Assays-By-Design Service; Life Technologies, Foster City, CA). Sequences of primers and probes were: 5'- ACCATTTATTTGATAGTCGGCACACT -3' (forward); 5' CTCCATGATGATTCTTTCCCTTTGATACTT -3' (reverse); 5'- AAGAGAAAACAAAGCACTGC -3' (VIC; specific to the G allele) and 5'- AAGAGAAAACAAAACACTGC -3' (FAM, specific to the A allele). Reactions were conducted in 96-well plates, in a total 5 µl 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 thermal cycler (7500 Fast Real-Time PCR System; Life Technologies) and heated for 10 min at 95°C, followed by 50 cycles of 95°C for 15 s and 63°C for 1 min. The genotyping success rate was better than 95%, with a calculated error rate based on PCR duplicates of less than 1%.

Isolation of mononuclear cells from peripheral blood of patients with T1DM and RNA extraction Samples of 10 ml peripheral blood were collected from a sub-sample of 26 T1DM patients belonging to our case-control study. Immediately after collecting the samples, an aliquot of 2 ml of blood was mixed with an equal volume of PBS. Then, total mononuclear cells were isolated from blood by density gradient centrifugation using Ficoll-paque^{TM} plus reagent (GE HealthCare, Uppsala, Sweden). Isolated mononuclear cells were stored at -80°C until RNA extractions.

Total RNA was extracted from mononuclear cells using the RNeasy Mini kit (Qiagen, Hilden, Germany). The concentration and quality of total RNA samples were assessed using a NANODROP 2000 spectrophotometer (Thermo Scientific Inc., Newark, DE). Only RNA samples with adequate purity ratios (A260/A280 = 1.9-2.1) were used for subsequent analyses [25]. In addition RNA integrity and purity were also

checked on agarose gel containing GelRed Nucleic Acid Gel Stain (Biotium Inc., Hayward, CA).

Quantification of IFIH1 gene expression by Real-Time PCR Real-Time PCR was performed in two separate reactions: first, RNA was reverse transcribed into cDNA, then cDNA was amplified by quantitative real-time PCR (RT-qPCR). Reverse transcription of 1 μg of RNA into cDNA was carried out using the High Capacity cDNA Reverse Transcription Kit (Life Technologies), following the manufacturer's protocol for oligo (dT) method.

RT-qPCR experiments were performed in a ViiA™ 7 Real-Time PCR System (Life Technologies). Experiments were performed by real-time monitoring of the increase in fluorescence of SYBER® Green dye [26]. Primers for the target (*IFIH1*) and reference (*GAPDH)* genes were: *IFIH1* 5'- ATGGAAAAAAAAGCTGCAAAAGA -3' (forward), *IFIH1* 5'-GTACTTCCTCAAATGTTCTGCACAA -3' (reverse), *GAPDH* 5'- ACCCACTCCTCCACCTTTG – 3' (forward) and *GAPDH* 5'- CTCTTGTGCTCTTGCTGGG – 3' (reverse). PCR reactions were performed using 5 μl of 2x Fast SYBER® Green Master Mix (Life Technologies), 0.5 μl (0.5 ng/μl) of forward and reverse primers for *IFIH1* or *GAPDH*, and 0.5 μl of cDNA template (1μg/μl), in a total volume of 10 μl. Each sample was assayed in triplicate and a negative control was included in each experiment. The thermocycling conditions for these genes were as follows: an initial cycle of 95°C for 20 s, followed by 50 cycles of 95°C for 5s and 60°C for 1 min. RT-qPCR specificity was determined using melting curve analyses and all primers generated amplicons that produced a single sharp peak during the analyses.

Quantification of the *IFIH1* cDNA was performed using the relative standard curve method [25, 27], and the *GAPDH* gene as the reference gene. Relative standard curves were generated for both target and reference genes by preparing serial dilutions of the same cDNA sample with a known relative quantity. Then, relative amounts of each *IFIH1* cDNA sample were obtained by normalizing their signals by those of *GAPDH* gene, and are presented as arbitrary units (AU).

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

The magnitude of associations between rs1990760 genotypes and T1DM or its categorical clinical characteristics were estimated using OR (95% CI). Multivariate logistic regression analyses were performed to assess the independent association of the rs1990760 polymorphisms with T1DM or its categorical clinical characteristics and to control for possible confounding factors whenever a statistically significant association was detected by univariate analyses.

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 performed using SPSS version 18.0 (SPSS, Chicago, IL).

Power calculations (PEPI program, version 4.0) showed that this study has a power of approximately 80% at a significance level of 0.05 to detect an odds ratio of 1.45 (for the presence of the A allele).

Results

Sample description The main clinical and laboratory characteristics of the 527 T1DM patients belonging to the present study were as follows: mean age was 33.6 ± 11.8 years; mean age at T1DM diagnosis was 17.4 ± 10.2 years; mean HbA1c was 8.7 ± 4.1% (71 ± 20 mmol/mol); and mean BMI was 24.7 ± 4.3 kg/m^2. Males comprised 48.0% of the sample, 7.2% were black, 28% of all patients had AH, 45.1% had some degree of DR, and 35.2% had some degree of DN.

Study of the association between the IFIH1 rs1990760 (G/A) polymorphism and type 1 diabetes mellitus or its clinical and laboratory characteristics Genotype and allele frequencies of the rs1990760 (G/A) polymorphism in T1DM patients and non-diabetic subjects are depicted in **Table 1.** Neither genotype nor allele frequencies of the rs1990760 polymorphism differed statistically between T1DM patients and non-diabetic subjects (P = 0.139 and P = 0.129, respectively), and all genotypes were in agreement with those predicted by the HWE in the two samples (P > 0.05). After adjustment for ethnicity, the A allele showed only a trend towards association with T1DM (A/G genotype: OR = 1.403, P = 0.058; A/A genotype: OR = 1.453, P = 0.061). However, the presence of the A allele was significantly associated with risk to T1DM under a dominant model of inheritance, adjusting for ethnicity (OR = 1.421, P = 0.037) (Table 1).

Clinical and laboratory characteristics of T1DM patients broken down by the different genotypes of the rs1990760 polymorphism are shown in **Table 2**. Age, age at T1DM diagnosis, proportion of males, BMI, creatinine levels, serum total cholesterol, triglycerides, HbA1c and presence of DR or DN did not differ statistically among the three genotypes. Interestingly, T1DM patients carrying the A allele showed lower levels of systolic BP as compared to patients homozygous for the G allele (A/A: 117.4 ± 16.7, A/G: 121.1 ± 19.0, G/G; 128.5 ± 18.9 mmHg; P = 0.001), taking into consideration a Bonferroni threshold of 0.0038. After Bonferroni correction, patients carrying the A/A or G/A genotypes also had lower levels of diastolic BP than patients with the G/G genotype (A/A: 74.4 ± 9.8, G/A: 78.0 ± 10.8, G/G: 82.4 ± 13.5 mmHg; P = 1 x 10^{-10}). It is worth noting that the exclusion of black patients from all these comparisons did not significantly change the data presented in Table 2.

To investigate the association between the rs1990760 (G/A) polymorphism and systolic and diastolic BP in greater depth, we further analyzed systolic and diastolic BP levels according to different genetic inheritance models: additive, recessive or dominant [28] (**Table 3**). When taking into account the additive model, both systolic and diastolic BP were lower in A/A genotype carriers as compared to G/G genotype carriers, adjusting for age, gender, ethnicity, presence of DN and treatment for hypertension (systolic BP: Beta = -13.972, P = 0.034, and diastolic BP: Beta = -12.186, P = 0.013). In the same way, when taking into account the dominant model, A allele carriers showed lower levels of both systolic and diastolic BP as compared to G/G genotype carriers, adjusting for age, gender, ethnicity, presence of DN and treatment for hypertension (systolic BP: Beta = -15.814, P = 0.019, and diastolic BP: Beta = -11.771, P = 0.003). Systolic and diastolic BP did not differ statistically among the rs1990760 genotypes under a recessive inheritance model (**Table 3**).

Genotype frequencies of the *IFIH1* polymorphism in T1DM patients according to the presence of AH are shown in **Figure 1**. The prevalence of AH was lower in T1DM patients carrying the A/A genotype as compared to patients with the G/G genotype (16.1% *vs*. 45.3%; adjusted residuals $P < 0.010$). The association of the A/A genotype with protection to AH was confirmed after adjustment for age, gender, presence of DN and ethnicity (OR = 0.339 for the A/A genotype, P = 0.019). Taking into account that ADA guidelines suggest that the cutoff for the diagnosis of AH in diabetic patients should be BP levels >130/80 mmHg [29], we reanalyzed our data using these values. In agreement with the results described above, the A/A genotype remained significantly associated with protection to AH under an additive model of inheritance and after adjustment for covariates (OR = 0.334, 95% CI = 0.180-0.622, P = 0.003).

In addition, we analyzed 725 patients with T2DM to know better whether the association of the rs1990760 A allele with systolic and diastolic BP was specific to T1DM or could be observed in a non-autoimmune diabetes context. Systolic BP did not differ significantly among T2DM patients broken down by the different genotypes of the rs1990760 polymorphism, adjusting for age, sex, ethnicity, BMI and treatment for hypertension (A/A: 145.7 ± 22.7, A/G: 141.5 ± 23.0, G/G; 144.5 ± 24.2 mmHg; P = 0.755). Diastolic BP in these patients also was similar among the three genotypes and controlling for the same covariates (A/A: 85.6 ± 12.9, G/A: 85.3 ± 13.9, G/G: 86.1 ± 14.1 mmHg; P = 0.800) (**Supplementary Table 1**). Accordingly, prevalence of AH also did not differ among different genotypes after adjustment for covariates (P = 0.163; **Supplementary Table 1**).

IFIH1 gene expression in mononuclear cells from a sub-sample of T1DM patients The median (minimum – maximum values) of *IFIH1* cDNA concentrations in mononuclear

cell samples from 26 T1DM patients was 6.3 (1.3 – 7.1) AU. *IFIH1* gene expression in this sample did not differ significantly among the three genotypes of the rs11990760 polymorphism [G/G (n = 7): 5.3 (1.4-6.8), G/A (n = 13): 6.6 (1.3-7.1), A/A (n = 7): 1.8 (1.3-6.7) AU; P = 0.274]. Nevertheless, *IFIH1* cDNA concentrations were increased in mononuclear cells from T1DM patients with AH (n=7) as compared with T1DM patients without AH (n=19) [6.7 (1.7-2.0) *vs*. 1.8 (1.3-7.0 AU), respectively; P = 0.036; **Figure 2**].

Discussion

In response to viral infections, the CARD domain of IFIH1 receptor associate with the CARD-containing adaptor molecule known as IPS-1 (IFN promoter stimulator-1), activating the transcription factors NF-κB and IRF-3, which then cooperate in induction of antiviral IFN-I response [13, 14, 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; thus, predisposing for T1DM developing [31].

An association between the *IFIH1* gene and T1DM was first reported by Smyth *et al*. [32], who performed a genome-wide association scan in European families with T1DM. Several polymorphisms located within the *IFIH1* gene region showed an association with T1DM, with the rs1990760 polymorphism being the most strongly associated with the disease (OR = 0.86, P = 1.4 x 10^{-10} for the G allele). This association between the rs1990760 polymorphism and T1DM was replicated in other populations [16, 17, 33, 34], and a recent meta-analysis comprising six different populations of European ancestry provided additional evidence for an influence of the A allele on

T1DM risk [34]. In the present study, we were able to replicate the reported association between the rs1990760 A allele and risk for T1DM.

The exact mechanisms by which IFIH1 polymorphisms contribute to T1DM pathogenesis remain to be explored. The rs1990760 polymorphism is not located in any functional region of the IFIH1 receptor, but the G allele is highly conserved among mammals and may have other, yet-unknown functions or may affect active domains through effects on the tertiary structure. Liu *et al*. [17] reported that the G/G genotype of this polymorphism was associated with 1.2-2.0 fold-increase in *IFIH1* gene expression in mononuclear cells of 374 subjects. Although our present data showed that *IFIH1* expression is 3.5 fold higher in mononuclear cells from T1DM patients carrying the G/G genotype when compared with A/A genotype patients, this difference did not reach formal statistical significance probably due to the small sample size analyzed. McCartney *et al*. [35] reported that optimal functioning of IFIH1 and prompt IFN-I response are required to prevent diabetes in C57BL/6 mice infected with encephalomyocarditis virus strain D, which has tropism for pancreatic beta-cells. Taking these data into consideration, we therefore hypothesize that probably due to a lower *IFIH1* expression, A/A genotype carriers might have a suboptimal IFIH1 function and, consequently, might be more predisposed to develop T1DM.

Our present results showed that age, age at T1DM diagnosis, proportion of males, BMI, creatinine levels, lipid profile, HbA1c and presence of DR or DN did not differ among the three genotypes of the rs1990760 polymorphism. Surprisingly, we observed that T1DM patients carrying the A allele had lower levels of both systolic and diastolic BP as compared to patients with the G/G genotype under additive and dominant models of inheritance. The A/A genotype was also associated with significant

protection for AH. To our knowledge, this is the first time that an *IFIH1* polymorphism is reported as being associated with AH.

Hypertension is a common disorder with uncertain etiology [36]. However, it is well known that vascular inflammation characterized by infiltration of immune cells is an important mechanism in the development of this condition [37]. The contribution of inflammation to the pathophysiology of high BP is supported by studies showing increased expression of adhesion molecules and their ligands, leukocyte extravasation, cytokine production, increased oxidative stress, and activation of immune cells and proinflammatory signaling pathways in arteries from hypertensive patients and rodents [37-39].

In the last years, it has become evident that components of both the innate and adaptive immune systems play an important role in hypertension [36, 40]. Macrophages and T-lymphocytes infiltrate in perivascular fat, heart and kidney of hypertensive patients and in rodents with experimental organ damage [36]. Interestingly, in mice lacking vascular macrophages, angiotensin-II and deoxycorticosterone acetate (DOCA)-salt treatment is unable to raise BP or induce oxidative stress or the vascular remodeling that follows the hypertensive stimulus [41, 42], corroborating the role of innate immunity in hypertension. Although these studies undoubtedly implicate macrophages and other components of innate immunity in the development of hypertension, the exact mechanisms by which they act remain uncertain. Harrison *et al*. [36] suggest that these mechanisms probably involve products of macrophages, including oxygen reactive species (ROS) and proinflammatory cytokines, which will diffuse to the adjacent endothelial cells and vascular smooth cells to alter their functions. This might induce activation of ROS-generating enzymes in these cells, stimulation of vascular smooth muscle hypertrophy and growth, production of vascular chemokines, cytokines and

adhesion molecules, alterations of nitric oxide production, and other alterations of vascular cell signaling. Moreover, similar action of ROS and proinflammatory cytokines on renal epithelial cells could also promote sodium and volume retention, contributing to hypertension [36].

Therefore, taking into account the role of low-grade inflammation in the pathogenesis of hypertension [36-38, 43, 44], our present results showing an association between the rs1990760 A allele and protection for AH seem to be biologically plausible albeit not yet completely understood. Recently, Chatterjee *et al*. [45, 46] demonstrated that all the three dsRNAs receptors, TLR-3, RIG-I and IFIH1, are activated excessively in placentas of mice and women with preeclampsia (PE), a pregnancy-specific syndrome characterized by excessive maternal immune system activation, inflammation, and endothelial dysfunction, causing hypertension. Chatterjee *et al*. [45] also showed that treatment of mice with intracellular synthetic dsRNA (PIC) significantly increased TLR3 levels and caused pregnancy-dependent hypertension, endothelial dysfunction and placental inflammation. Our results demonstrating that *IFIH1* gene expression is increased in mononuclear cell from T1DM patients with AH is in agreement with the data of Chatterjee *et al*. [45, 46] indicating that high expression of dsRNA receptors have a role in hypertension. Therefore, it seems reasonable to presume that T1DM patients carrying the A/A genotype may have a lower risk for developing AH due to a decreased *IFIH1* gene expression.

Even though the intermediate signaling cascade of RIG-I and IFIH1 are different from TLR-3, they converge to activate the transcription factor NF-κB, which will induce the IFN-I response, which is responsible for eliciting antiviral response and inflammation [13, 14]. An environment rich in proinflammatory cytokines will promote an accumulation of immune cells that will produce more inflammatory cytokines and

exacerbate the inflammatory cascade, which under certain circumstances may contribute to mechanisms that lead to hypertension and end-organ damage [38]. Further studies are needed to elucidate which are the ligands that stimulated dsRNA receptors in PE and AH. Apart from recognizing dsRNA from viruses, TLR3, RIG-I and IFIH1 can be also activated by a number of additional molecules including small self-RNA and damage-associated-molecular patterns released from dead or damaged tissue during stress or apoptosis (reviewed in [46]). Our data indicating that the *IFIH1* rs1990760 polymorphism is associated with AH in T1DM patients but not in patients with T2DM suggest that the role of this gene in hypertension is dependent of an immune attack as occurring in the development of T1DM or PE.

In conclusion, our results confirm the association between the A allele of the *IFIH1* rs1990760 polymorphism and T1DM in our population. Moreover, the A allele seems to be associated with a significant protection for AH in T1DM patients. Additional studies are necessary to confirm the association of the rs1990760 polymorphism with protection for AH in other populations and to evaluate which ligand activates the IFIH1 receptor and how increased levels of this dsRNA receptor lead to AH.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement A.P.B. conceived and designed the study, collected and analyzed data and wrote the first draft of the manuscript; F.S.O. collected and analyzed data, contributed to the discussion and reviewed the final version of the manuscript; L.A.B. analyzed data, contributed to the discussion and reviewed the final version of the manuscript; N.E.L. collected and analyzed data, contributed to the discussion and reviewed the final version of the manuscript; L.H.C. contributed to the analyses of data and to the discussion and reviewed the final version of the manuscript; D.C. conceived and designed the study, analyzed data and wrote the manuscript.

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Table 1 Genotype and allele frequencies of the *IFIH1* rs1990760 G/A polymorphism in patients with type 1 diabetes mellitus (T1DM) and non-diabetic subjects.

	T1DM patients (n= 527)	Non-diabetic subjects (n= 517)	Unadjusted P*	Adjusted OR (95% CI) / P†
Genotype				
A/A	150 (28.5%)	139 (26.9%)	0.139	1
G/A	263 (49.9%)	239 (46.2%)		1.403 (0.989-1.991) / 0.058
G/G	114 (21.6%)	139 (26.9%)		1.453 (0.983-2.147) / 0.061
Allele				
A	0.534	0.500	0.129	
G	0.466	0.500		
Additive model				
A/A	150 (56.8%)	139 (50.0%)	0.133	1
G/G	114 (43.2%)	139 (50.0%)		1.471 (0.989-2.187) / 0.056
Recessive model				
A/A	150 (28.5%)	139 (26.9%)	0.617	1

G/A-G/G	377 (71.5%)	378 (73.1%)		1.157 (0.850-1.575) / 0.354
Dominant model				
G/A-A/A	413 (78.4%)	378 (73.1%)	0.048	1
G/G	114 (21.6%)	139 (26.9%)		1.421 (1.022-1.975) / 0.037

Data are presented as number (%) or proportion.

* P values were computed by χ^2 tests comparing T1DM patients and non-diabetic subjects.

†Adjusted OR (95% CI) and P values obtained by logistic regression analysis controlling for ethnicity.

Table 2 Clinical and laboratory characteristics of patients with type 1 diabetes mellitus, broken down by the different genotypes of the *IFIH1* rs1990760 (G/A) polymorphism.

	***IFIH1* rs1990760 (G/A) polymorphism**			
	G/G	**G/A**	**A/A**	P*
	(n = 114)	**(n = 263)**	**(n = 150)**	
Age (years)	36.1 ± 12.6	34.9 ± 14.6	32.8 ± 12.5	0.356
Gender (% male)	51.1	50.5	56.2	0.581
Age of diagnosis (years)	19.2 ± 9.9	16.5 ± 9.7	16.7 ± 11.0	0.298
BMI (kg/m^2)	22.5 ± 6.5	23.8 ± 4.4	22.2 ± 4.9	0.088
Diabetic nephropathy (%)	35.3	30.3	28.0	0.679
Diabetic retinopathy (%)	50.0	43.7	39.4	0.352
Systolic blood pressure (mm/Hg)†	128.5 ± 18.9 [a]	121.1 ± 19.0 [b]	117.41 ± 16.7 [b]	0.001
Diastolic blood pressure (mm/Hg)†	82.4 ± 13.5 [a]	78.0 ±10.8 [b]	74.4 ± 9.8 [c]	1×10^{-10}
Triglycerides (mmol/L)	0.86 (0.93-1.48)	0.94 (1.08-1.46)	0.84 (0.90-1.98)	0.248
Creatinine (µmol/L)	88.4 (97.2-185.6)	88.4 (88.4-123.7)	79.6 (79.6-114.9)	0.265

HbA1c (% [mmol/mol])	9.0 ± 2.2 [75 ± 0.5]	8.6 ± 2.2 [70 ± 0.5]	9.24 ± 7.0 [77 ± 53]	0.465
HDL cholesterol (mmol/L)	1.5 ± 0.6	1.5 ± 0.4	1.5 ± 0.5	0.664
Total cholesterol (mmol/L)	4.7 ± 1.2	4.8 ± 1.2	4.5 ± 1.1	0.184

Data are expressed as mean ± SD, median (minimum–maximum values), or percentage.

*P-values were obtained by One-Way ANOVA or χ^2 tests, as appropriate.

n = number of subjects. HbA1c = glicohemoglobin.

Only P values lower than the Bonferroni threshold (P = 0.0038) were considered statistically significant.

† Different letters mean that values were significantly different using Tukey's post hoc test ($P < 0.05$).

Table 3 Systolic and diastolic blood pressure levels in T1DM patients broken down by different inheritance models of the *IFIH1* rs1990760 (G/A) polymorphism

Models	Systolic BP	Unadjusted P*	Beta / adjusted P†	Diastolic BP	Unadjusted P*	Beta / adjusted P†
Additive model						
G/G	128.5 ± 18.9	$1x10^{-10}$	-14.384 / 0.034	82.4 ± 13.5	$1x10^{-10}$	-12.525 / 0.013
A/A	117.4 ± 16.7			74.4 ± 9.8		
Recessive model						
G/A-G/G	123.5 ± 19.2	0.007	-2.271 / 0.717	79.4 ± 11.9	$1x10^{-10}$	-3.512 / 0.361
A/A	117.4 ± 16.7			74.4 ± 9.8		
Dominant model						
G/G	128.5 ± 18.9	$1x10^{-10}$	-15.649/ 0.019	82.4 ± 13.5	$1x10^{-10}$	-12.155 / 0.003
G/A-A/A	119.7 ± 18.2			76.6 ± 10.6		

Data are expressed as mean ± SD.

*P-values were obtained by Student's t-test.

† Beta / Adjusted P values were obtained from multiple linear regression analyses adjusting for age, sex, ethnicity, presence of diabetic nephropathy and treatment for hypertension.

T1DM = type 1 diabetes mellitus; BP = blood pressure.

Only unadjusted P values lower than the Bonferroni threshold (P = 0.0083) were considered statistically significant.

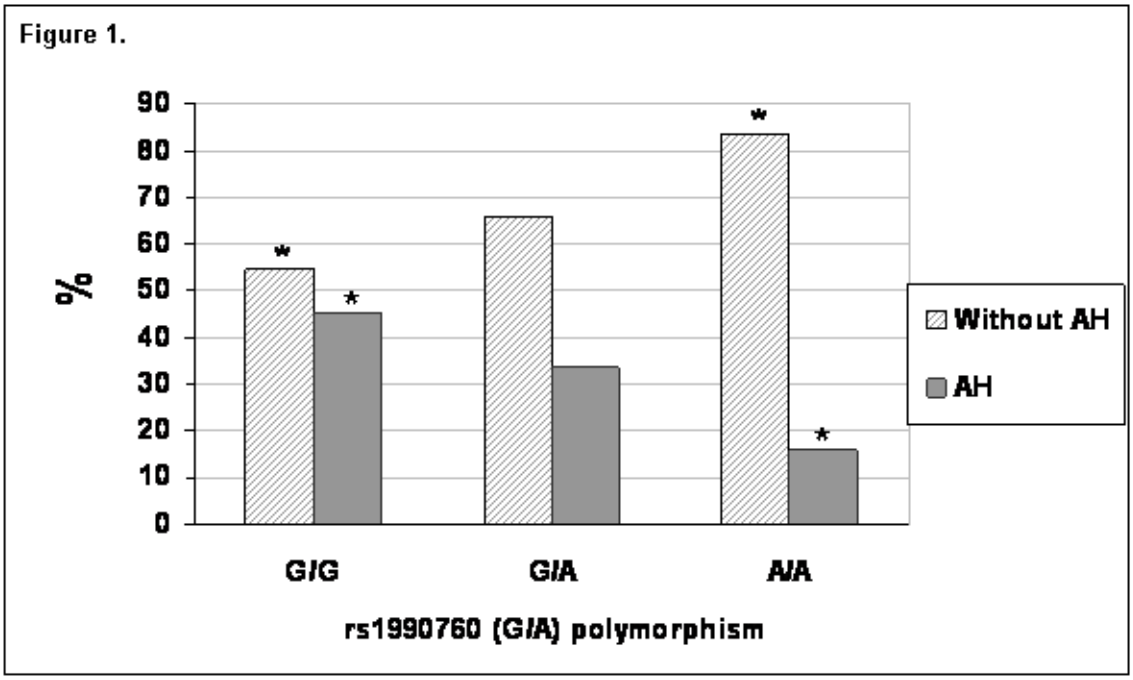

Figure 1 Genotype frequencies of the *IFIH1* rs1990760 (G/A) polymorphism according to the presence of systemic arterial hypertension (AH) in patients with type 1 diabetes mellitus ($P = 1 \times 10^{-10}$). The association with AH was confirmed after adjustment for age, gender, presence of diabetic nephropathy and ethnicity (OR = 0.339 for the A/A genotype; 95% CI = 0.14-0.84; P = 0.019). * Significant differences at $P < 0.01$ (adjusted residuals).

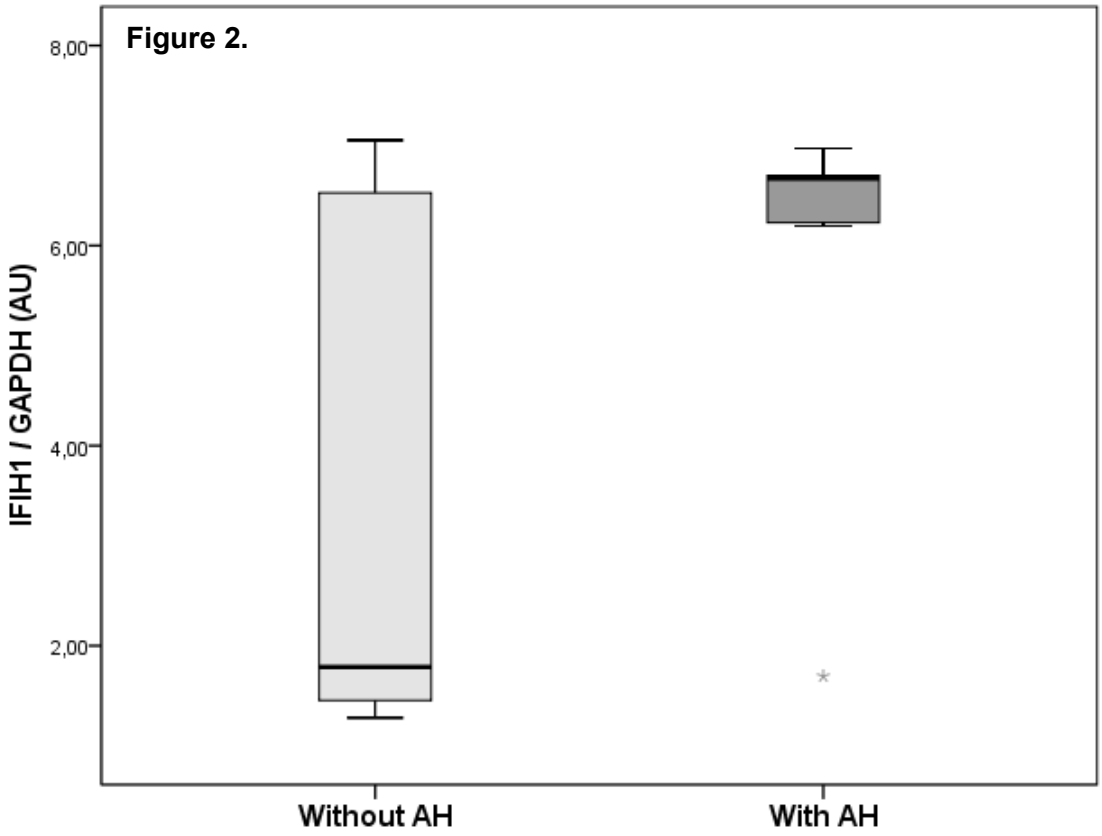

Figure 2 *IFIH1* gene expressions in mononuclear cells from type 1 diabetic patients broken down by the presence of arterial hypertension (AH) (P = 0.036). P values were obtained using Student-s t-test. Data are presented as median (95% CI). AU = arbitrary units.

Supplementary Table 1. Clinical and laboratory characteristics of patients with type 2 diabetes mellitus, broken down by the different genotypes of the *IFIH1* rs1990760 (G/A) polymorphism.

	***IFIH1* rs1990760 (G/A) polymorphism**			
	G/G	**G/A**	**A/A**	Adjusted P *
	(n = 221)	**(n = 301)**	**(n = 203)**	
Systolic blood pressure (mm/Hg)	144.5 ± 24.2	141.5 ± 23.0	145.7 ± 22.7	0.755
Diastolic blood pressure (mm/Hg)	86.1 ± 14.1	85.3 ± 13.9	85.6 ± 12.9	0.800
Systemic arterial hypertension (%)	30.3	41.7	28.0	0.163

Data are expressed as mean ± SD. *Adjusted P values were obtained from multiple linear regression analyses adjusting for age, sex, ethnicity and treatment for hypertension. BP = blood pressure.