The rs1893217 (T/C) polymorphism in *PTPN2* gene is not associated with type 1 *diabetes mellitus* in subjects from Southern Brazil

O polimorfismo rs1893217 (T/C) no gene PTPN2 *não está associado com diabetes melito em indivíduos do Sul do Brasil*

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ABSTRACT

Objective: To evaluate the association of the *PTPN2* rs1893217 polymorphism with T1DM and/or its clinical and laboratory characteristics in a Caucasian population from Southern Brazil. **Subjects and methods:** Four hundred and eighty six patients with T1DM and 484 non-diabetic subjects were included in the study. Genotyping of the *PTPN2* rs1893217 was performed by real-time PCR. **Results:** Genotype frequencies did not differ between T1DM patients and non-diabetic subjects (P = 0.265). The C allele was observed in 14.5% of the T1DM sample and 12.2% of the non-diabetic group (P = 0.152). Moreover, the frequencies of this variant did not differ statistically between T1DM patients and non-diabetic subjects when assuming recessive, dominant, or additive inheritance models. The clinical and laboratory characteristics of T1DM patients did not differ significantly among the three genotypes of the rs1893217 polymorphism, either. **Conclusion:** The *PTPN2* rs1893217 polymorphism is not significantly associated with T1DM in Caucasian subjects from Southern Brazil. Arq Bras Endocrinol Metab. 2014;58(4):382-8

Keywords
Type 1 *diabetes mellitus*; polymorphism; *PTPN2* gene; rs1893217

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RESUMO

Objetivo: Avaliar a associação do polimorfismo rs1893217 no gene *PTPN2* com DM1 e/ou suas características clínicas e laboratoriais em uma população de brancos do Sul do Brasil. **Sujeitos e métodos:** Quatrocentos e oitenta e seis pacientes com DM1 e 484 indivíduos não diabéticos foram incluídos no estudo. A genotipagem do *PTPN2* rs1893217 foi realizada por PCR em tempo real. **Resultados:** As frequências genotípicas não diferiram entre os pacientes com DM1 e indivíduos não diabéticos (p = 0,265). O alelo C foi observado em 14,5% da amostra com DM1 e 12,2% no grupo de não diabéticos (p = 0,152). Além disso, as frequências dessa variante não diferiram estatisticamente entre os pacientes com DM1 e indivíduos não diabéticos considerando-se os modelos de herança recessivo, dominante ou aditivo. As características clínicas e laboratoriais dos pacientes com DM1 também não diferiram significativamente entre os três genótipos do polimorfismo rs1893217. **Conclusão:** O polimorfismo rs1893217 do gene *PTPN2* não está associado com DM1 em brancos do Sul do Brasil. Arq Bras Endocrinol Metab. 2014;58(4):382-8

Descritores
Diabetes tipo 1; polimorfismo; gene *PTPN2*, rs1893217

INTRODUCTION

Type 1 *diabetes mellitus* (T1DM) is a complex disease resulting from a cellular-mediated autoimmune destruction of pancreatic beta-cells, rendering patients insulin-dependent for life (1). Destruction of beta-cells arises from the interplay of multiple genetic and environmental risk factors, which are still poorly defined (2,3). The major susceptibility locus maps to the *HLA class II* genes (OR > 6) at chromosome 6p21, and accounts for 30-50% of the genetic risk for the condition (4). Genome-wide association studies allowed the identification of several non-HLA loci with smaller effects on the genetic predisposition for T1DM (2), including the insulin gene, the *CTLA4* gene, the *IL2RA* gene, the *IFIH1* gene, the *PTPN22* gene, and the *PTPN2* (*protein tyrosine phosphatase N2*) gene (5,6).

There is substantial evidence that viral pathogens, such as enteroviruses, play a major role in triggering the autoimmune destruction of pancreatic beta-cells (3). Type 1 interferons (IFNs) are important cytokines of the host immune response against viral infections (7). In addition to antiviral responses, these cytokines are able to induce apoptosis and to promote systemic autoimmunity (8). To control these effects of IFNs, organisms have developed several negative regulators of the IFN responses, and one of these regulators is PTPN2 (9).

The *PTPN2* gene is located on chromosome 18 and encodes a phosphatase that is a member of the first non-transmembrane (NT1) subfamily of protein tyrosine phosphatases (10). PTPN2 functions as a negative regulator of several signaling pathways, including Janus kinases (JAKs), signal transducer and activator of transcription (STATs), p42/44 MAPK (ERK) and epidermal growth factor receptor and insulin receptor β, also modulating beta-cell apoptosis induced by IFN-γ (11,12). Moreover, PTPN2 knockdown induces aberrant pro-apoptotic activity of the BH3-only protein Bim, resulting in increased apoptosis via JNK activation and the intrinsic apoptotic pathway in INS-1E, primary rat, and human beta-cells treated with type I or type II IFNs (13).

The Wellcome Trust Case Control Consortium (WTCCC) genome-wide association study reported an association of the rs2542151 single-nucleotide polymorphism (SNP), located 5.5kb upstream of the *PTPN2* gene, with three autoimmune diseases: Crohn's disease, rheumatoid arthritis, and T1DM (14). Todd and cols. (5) performed a follow-up study with the aim of replicating the WTCCC results on T1DM. The authors analyzed 11 SNPs within the disequilibrium block that contains the *PTPN2* gene, and reported that two of them, rs478582 in intron 3, and rs1893217 in intron 7, were independently associated with T1DM. Moreover, the rs1893217 SNP was in strong linkage disequilibrium with rs2542151 SNP (5). In addition, Long and cols. (15) showed that the C allele of the rs1893217 SNP is associated with decreased *PTPN2* expression in $CD4^+$ T cells. Therefore, the aim of this study was to replicate the reported association between the rs1893217 SNP and T1DM in an independent population from Southern Brazil.

SUBJECTS AND METHODS

Subjects and phenotype measurements

This was a case-control study designed to investigate whether the *PTPN2* rs1893217 SNP is associated with T1DM. The diabetic sample comprised 486 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 (1). The non-diabetic sample comprised 484 healthy blood donors who did not have *diabetes mellitus* or family history of this disease (mean age = 40.0 ± 7.8; male = 50.0%). All subjects were Caucasian Brazilians, and the ethnic group was self-reported.

All T1DM patients underwent physical and laboratory evaluations. They were weighed barefoot, wearing light outdoor clothes, and their height was measured as well. Body mass index (BMI) was calculated as weight (kg)/height (in meters) squared. Blood pressure (BP) was measured by a trained researcher with a mercury sphygmomanometer (Korotkoff phases I and V) using an appropriated cuff size, on the left arm of the patient 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 the initial visit, and at two follow-up visits within 1 month of the initial visit, or if the presence of AH was registered on the medical records. Assessment of diabetic retinopathy (DR) was performed in all patients by an experienced ophthalmologist using fun-

doscopy through dilated pupils. DR was classified using the scale developed by the Global Diabetic Retinopathy Group (16). 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) (17).

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. Glycated hemoglobin (HbA1c) measurements were performed by different methods, and results were traceable to the DCCT method. Total plasma cholesterol, HDL cholesterol, and triglycerides were assayed using enzymatic methods. Urinary albumin was measured by immunoturbidimetry (Microalb; Ames-Bayer, Tarrytown NY), and intra- and interassay coefficients of variation in our laboratory were both < 6%. The research protocol was approved by the Hospital ethics committee, and all subjects signed an informed consent form.

Genotyping

DNA was extracted from peripheral blood leukocytes using a standardized salting-out procedure. The *PTPN2* rs1893217 SNP 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' GGCCCAGATACACTCTTCTTCCT 3' (forward); 5' TGTGCTCTCACTTGTCACCATT 3' (reverse); 5' CCTAGGGACAGAGGTAG 3' (VIC) and 5' CTAGGGACAAAGGTAG 3' (FAM). 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 greater than 95%, with a calculated error rate based on PCR duplicates of less than 1%.

Statistical analysis

Allelic 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 patients using χ^2-tests. Clinical and laboratory characteristics were compared among groups using One-way ANOVA, Student's t-test or χ^2, as appropriate. In addition, General Linear Model (GLM) analyses were performed for comparing clinical and laboratory characteristics among genotypes adjusting for possible confounding factors. Variables with normal distribution are presented as mean ± SD or percentages. Variables with skewed distribution were log-transformed before analyses and are presented as medians (minimum-maximum values). The magnitude of associations between rs1893217 SNP and T1DM assuming different inheritance models were estimated using odds ratios (OR) with 95% CI.

Results for which P was less than 0.05 were considered statistically significant. Bonferroni corrections were 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 1.65 (for the presence of the C allele).

RESULTS

The main clinical and laboratory characteristics of the 486 T1DM patients belonging to the present study were as follows: mean age was 33.9 ± 13.1 years; mean age at T1DM diagnosis was 17.3 ± 10.1 years, mean HbA1c was 9.1 ± 5.6%, and mean BMI was 23.1 ± 4.8 kg/m^2. Males comprised 49.4% of the sample, 27.8% of all patients had AH, 43.0% had some degree of DR, and 32.3% had some degree of DN.

Genotype and allele frequencies of the *PTPN2* rs1893217 SNP (T/C) polymorphism in T1DM patients and non-diabetic subjects are depicted in table 1. Genotype frequencies of the rs1893217 SNP did not differ significantly between T1DM patients and non-diabetic subjects (P = 0.265), and they were in agreement with those predicted by the HWE in the two samples (P > 0.05). In the same way, the minor allele (C) of the rs1893217 SNP was similarly distributed between diabetic patients and non-diabetic subjects (14.2% *vs.* 12.2%, respectively; P = 0.152). The frequencies of this

Table 1. Genotype and allele frequencies of the *PTPN2* rs1893217 T/C polymorphism in patients with type 1 *diabetes mellitus* (T1DM) and non-diabetic subjects

	T1DM patients (n = 486)	Non-diabetic subjects (n = 484)	P value*
Genotype			
T/T	357 (73.5%)	377 (77.9%)	
T/C	117 (24.0%)	96 (19.8%)	0.265
C/C	12 (2.5%)	11 (2.3%)	
Allele			
T	0.855	0.878	0.152
C	0.145	0.122	
Recessive model			
C/C	12 (2.5%)	11 (2.3%)	0.99
T/C – T/T	474 (97.5%)	473 (97.7%)	
Dominant model			
T/C – C/C	129 (26.5%)	107 (22.1%)	0.125
TT	357 (73.5%)	377 (77.9%)	
Additive model			
C/C	12 (3.2%)	11 (2.8%)	0.903
T/T	357 (96.8%)	377 (97.2%)	

Data are presented as numbers (%) or proportions. * P values were computed by χ^2 tests comparing T1DM patients and non-diabetic subjects. Only P values lower than the Bonferroni threshold [P = 0.01 (0.05/5 comparisons)] were considered statistically significant.

variant also did not differ statistically between T1DM patients and non-diabetic subjects when assuming different genetic inheritance models: recessive (T/C + T/T *vs.* C/C), dominant (T/T *vs.* T/C + C/C), or additive (C/C *vs.* T/T) (Table 1).

Clinical and laboratory characteristics of T1DM patients broken down by the different genotypes of the rs1893217 SNP are shown in table 2. Mean age, proportion of males and age at T1DM diagnosis were similar among the different genotype of the rs1893217 SNP (P = 0.323, P = 0.164, and P = 0.182, respectively). Moreover, BMI, lipid and glucose profile, creatinine levels, systolic and diastolic BP, and presence of DR and DN also did not differ statistically among the three genotypes (Table 2). Clinical and laboratory characteristics of T1DM patients according to the presence of the minor allele (C) of the rs1893217 SNP (dominant model) are shown in table 3. In agreement with genotype data, none of these characteristics differed between patients carrying the C allele as compared with patients with the T/T genotype (Table 3). These characteristics were not compared using other inheritance model due to the small number of subjects carrying the C/C genotype (n = 12).

Table 2. Clinical and laboratory characteristics of patients with type 1 *diabetes mellitus* broken down by the different genotypes of the *PTPN2* rs1893217 (T/C) polymorphism

	***PTPN2* rs1893217 (T/C) polymorphism**			
	T/T (n = 357)	**T/C (n = 117)**	**C/C (n = 12)**	**P***
Age (years)	33.5 ± 11.8	35.8 ± 17.9	40.0 ± 13.9	0.323
Gender (% male)	54.4	44.3	54.5	0.164
Age of diagnosis (years)	16.8 ± 10.0	15.7 ± 9.0	23.3 ± 12.9	0.182
BMI (kg/m^2)	23.0 ± 5.1	24.0 ± 3.4	21.1 ± 5.7	0.256
Systolic BP (mmHg)	121.1 ± 17.3	123.3 ± 20.7	116.0 ± 11.5	0.500
Diastolic BP (mmHg)	78.0 ± 11.6	78.0 ± 13.1	71.4 ± 12.1	0.357
HDL cholesterol (mmol/L)	1.45 ± 0.45	1.49 ± 0.53	1.51 ± 0,38	0.950
Total cholesterol (mmol/L)	4.72 ± 1.29	4.60 ± 0.97	4.23 ± 0.70	0.362
Triglycerides (mmol/L)	0.85 (0.28 – 10.16)	0.92 (0.28 – 6.02)	0.66 (0.36 – 5.72)	0.680
Creatinine (µmol/L)	88.4 (35.36 – 981.24)	88.4 (44.12 – 934.0)	79.56 (53.04 – 247.52)	0.605
HbA1c (%)	8.9 ± 4.9	8.6 ± 2.2	8.7 ± 1.3	0.907
Diabetic nephropathy (%)	30.2	28.0	33.3	0.939
Diabetic retinopathy (%)	43.2	47.5	30.0	0.525

Data are presented as means ± SD, medians (minimum–maximum values) or %. BMI, body mass index; BP = blood pressure. HbA1c = glycohemoglobin. * P values were obtained using χ^2 or ANOVA tests, as appropriate. Only P values lower than the Bonferroni threshold [P = 0.0038 (0.05/13 comparisons)] were considered statistically significant.

Table 3. Clinical and laboratory characteristics of patients with type 1 *diabetes mellitus*, broken down by the presence of the minor allele (C) of the *PTPN2* rs1893217 (T/C) polymorphism

	***PTPN2* rs1893217 (T/C) polymorphism**		
	T/T (n = 357)	**T/C – C/C (n = 129)**	**P value***
Age (years)	33.5 ± 11.8	36.2 ± 17.5	0.186
Gender (% male)	54.4	44.4	0.099
Age at diagnosis (years)	16.8 ± 9.9	16.4 ± 9.6	0.758
BMI (kg/m^2)	23.0 ± 5.1	23.6 ± 3.8	0.381
Systolic BP (mm/Hg)	121.1 ± 17.3	122.6 ± 20.0	0.553
Diastolic BP (mm/Hg)	78.0 ± 11.6	77.4 ± 13.0	0.718
HDL cholesterol (mmol/L)	1.45 ± 0.45	1.50 ± 0.51	0.757
Total cholesterol (mmol/L)	4.72 ± 1.29	4.54 ± 0.95	0.237
Triglycerides (mmol/L)	0.85 (0.28 – 10.16)	0.86 (0.28-6.48)	0.421
Creatinine (µmol/L)	88.4 (35.36 – 981.24)	88.4 (44.2-937.04)	0.419
HbA1c (%)	8.9 ± 4.9	8.6 ± 2.1	0.660
Diabetic nephropathy (%)	30.2	28.6	0.953
Diabetic retinopathy (%)	43.2	45.6	0.789

Data are means ± SD, medians (minimum–maximum values) or %. BMI: body mass index. BP: blood pressure. HbA1c: glycohemoglobin. * P values obtained from Student's t-tests. Only P values lower than the Bonferroni threshold [P = 0.0038 (0.05/13 comparisons)] were considered statistically significant.

DISCUSSION

PTPN2 gene is expressed in several cell types, including beta-cells, intestinal epithelial cells, and immune cells (12). Transfection with siRNAs against *PTPN2* inhibits basal and cytokine-induced *PTPN2* expression in rat beta-cells and dispersed human islet cells. Decreased *PTPN2* expression exacerbates IL-1β + IFN-γ-induced beta-cell apoptosis and turns IFN-γ alone into a pro-apoptotic signal. Moreover, inhibition of *PTPN2* amplifies IFN-γ-induced STAT-1 phosphorylation, whereas double knockdown of both *PTPN2* and *STAT-1* protects beta-cells against cytokine-induced apoptosis, suggesting that STAT-1 hyperactivation is responsible for the aggravation of cytokine-induced beta-cell death in PTPN2-deficient cells (12). Therefore, PTPN2 may contribute to the pathogenesis of T1DM since changes in PTPN2 function in beta-cells sensitize these cells to pro-apoptotic inflammatory signals, potentially amplifying beta-cell loss and insulitis, independent of its potential effects on the immune system (12).

In agreement with a potentially important role of PTPN2 in T1DM pathogenesis, genome-wide association studies have reported an association between *PTPN2* polymorphisms and T1DM (2,5,14). However, in the present case-control study, we were not able to replicate the reported association of the rs1893217 SNP in the *PTPN2* gene with T1DM in Caucasian Brazilians. One possible explanation for the discrepant results is that most of T1DM studies selected their sample population limiting the age at T1DM onset to less than 17 years to avoid including type 2 diabetes misdiagnosed patients. This was the strategy used for the above mentioned genome-wide scan studies, but it was not an exclusion criteria used in our study since the mean age at diagnosis of our patients was 17.3 ± 10.1 years. We decided not exclude patients with age of onset higher than 17 years because T1DM diagnosis may happen at any age; thus, excluding this group of patients could prevent us from determining the possible different genetic background between early and late-onset T1DM subgroups. In this context, Espino-Paisan and cols. (18) showed that the frequency of the rs2542151 G allele of the *PTPN2* gene was significantly higher in an early-T1DM onset group (onset before 16 years) compared with late-onset T1DM patients and controls (OR = 1.61; 95% CI 1.14-2.26). No significant differences were found between controls and late-onset group. It is worth noting that, in the present study, the frequencies of the *PTPN2* rs1893217 SNP were similar between early- and late-T1DM onset patients (P > 0.05); therefore, age of T1DM onset might not have influenced our results.

As already mentioned, the strongest genetic association for T1DM is with the HLA class II genes (4). Consequently, cohort studies following children

with high-risk HLA genotypes to islet autoimmunity and T1DM development offers a valuable tool to further validate the independent predictive value of non-HLA loci and to explore the genetic architecture of T1DM in different populations. Steck and cols. (6) assessed the effects of 20 non-HLA gene polymorphisms on the risk of islet autoimmunity and progression to T1DM in the Diabetes Autoimmunity Study in the Young (DAISY). Interestingly, they observed that the *PTPN2* rs1893217 SNP predicted islet autoimmunity [Hazard ratio (HR) 1.42, 95% CI 1.02–1.99] but not T1DM development, after controlling for family history of T1DM and HLA high-risk genotypes (6). The absence of association with T1DM is in agreement with the present study. Unfortunately, we did not evaluate HLA high-risk genotypes in our population to know if this genetic background would modify our results.

Interactions with non-HLA genes also might influence the effects of the *PTPN2* rs1893217 SNP on T1DM risk. For example, Frederiksen and cols. (19) reported an interaction between vitamin D receptor (*VDR*) and *PTPN2* genes on the progression to T1DM in children from the DAISY cohort: in children with the *PTPN2* rs1893217 A/A genotype, the *VDR* rs1544410 A allele was associated with a decreased risk of T1DM (HR = 0.24, 95%CI 0.11-0.53), while in children with the *PTPN2* rs1893217 G allele, the *VDR* rs1544410 A allele was not associated with this disease. This interaction has biological plausibility, since the VDR binds to a novel intronic binding site in the *PTPN2* gene (20), and suggests that variations in both *VDR* and *PTPN2* genes are necessary to lead to an effect on T1DM risk.

Some factors unrelated to the *PTPN2* rs1893217 SNP 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 Caucasian-Brazilian 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 *PTPN2* rs1893217 SNP and T1DM. Power calculations were based on OR of 1.65 for the presence of the C allele; however, we had less than 80% power to detect OR ≤ 1.65, which is the case for most of the T1DM-related genes (5). Third, interactions of the analyzed polymorphism with other important characteristics, such as islet autoantibodies and HLA high-risk genotypes could have influenced our results; unfortunately, we do not have information about these covariates in our sample.

In conclusion, the present study found no evidence for a significant association between the rs1893217 SNP in the *PTPN2* gene and T1DM. Further multicenter studies including larger sample numbers and analyzing gene-gene interactions are necessary to confirm these results in the Brazilian population. Furthermore, prospective studies following children since islet autoimmunity development to progression to T1DM are also necessary, as they may offer additional insights concerning the association of *PTPN2* and other T1DM-related genes with T1DM.

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