



## Association of *TNF- $\alpha$* , *CTLA4*, and *PTPN22* polymorphisms with type 1 diabetes and other autoimmune diseases in Brazil

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**ABSTRACT.** Type 1 diabetes mellitus (T1D) is a complex disorder characterized by an autoimmune response against human pancreatic beta-cells. Patients with T1D can also develop a response toward one or more other factors, such as in autoimmune thyroiditis (AITD) and celiac disease (CD). In the presence of T1D + AITD, the patient is diagnosed with autoimmune polyglandular syndrome type III (APSIII); patients with APSIII may also present with CD. These diseases have a strong genetic component and share many susceptibility genes, suggesting potentially overlapping pathogenic pathways. Polymorphisms in the *TNF- $\alpha$*  (rs1800629), *CTLA-4* (rs231775), and *PTPN22* (rs2476601) genes have been previously associated with T1D; however, there is no consensus regarding their role in

T1D and scarce literature focusing on AITD and/or CD. Thus, we analyzed these genetic variants in 205 Northeast Brazilian patients with T1D and with/without AITD and/or CD, and in 308 healthy controls. The *PTPN22* gene variants were associated with T1D susceptibility and APSIII [odds ratio (OR) = 2.57 and 2.77, respectively]. *CTLA4* rs231775 and *TNF- $\alpha$*  rs1800629 were not associated with T1D onset in the Brazilian population. However, when comparing APSIII individuals in the T1D only group, we observed an association of the *TNF- $\alpha$*  SNP in the allelic ( $P = 0.0442$ ; OR = 0.44) and dominant models ( $P = 0.0387$ ; OR = 0.40). This study reinforces the importance of *CTLA-4* and other variants in unraveling the pathogenic mechanisms of T1D in different populations and in understanding their relationships with the development of other T1D-related autoimmune diseases.

**Key words:** Type 1 diabetes mellitus; Autoimmune disease; *CTLA-4*; *PTPN22*; *TNF- $\alpha$*

## INTRODUCTION

Type 1 diabetes mellitus (T1D) is caused by an autoimmune reaction with both genetic and environmental factors and promotes the destruction of insulin-producing pancreatic beta cells (Sugihara, 2012). The genetic component is crucial to T1D onset and has been the subject of intensive study during the last four decades (Noble and Erlich, 2012). These studies have revealed the human leukocytes antigen (HLA) encoding genes as the main locus associated with T1D onset, corresponding to over 40% of disease susceptibility (Steck and Rewers, 2011; Noble and Erlich, 2012). However, more than 40 loci have been described that also contribute to T1D pathogenesis (Bergholdt et al., 2012; Sugihara, 2012). Among these, three genetic variants in the *CTLA-4* (rs231775), *PTPN22* (rs2476601), and *TNF- $\alpha$*  (rs1800629) genes have been previously associated not only with T1D, but also with different autoimmune disorders including autoimmune thyroid disease (AITD) (Luo et al., 2012; Pan and Xing, 2012; Pastuszak-Lewandoska et al., 2012), celiac disease (CD) (Ueda et al., 2003; Eyre et al., 2010), systemic lupus erythematosus (SLE) (Pradhan et al., 2010), and rheumatoid arthritis (RA) (Nong et al., 2011), albeit in different populations.

Some diabetic patients also develop AITD and/or CD, at a higher frequency than in the general population (Witek et al., 2012): the prevalence of CD + T1D ranges between 4.4-11.1% versus 0.5% in the common population (Camarca et al., 2012) and 15-30% of patients with T1D also have AITD (Van den Driessche et al., 2009). In the presence of both T1D + AITD, a patient is diagnosed with autoimmune polyglandular syndrome type III (APSIII) (Horie et al., 2012; Wémeau et al., 2013). Furthermore, patients with APSIII can also present with CD. It is logical therefore to suggest that different autoimmune disorders potentially share common pathogenetic pathways and that the *TNF- $\alpha$* , *CTLA-4*, and *PTPN22* genes might be involved in these common underlying mechanisms.

The tumor necrosis factor alpha (*TNF- $\alpha$* ) gene, located on chromosome 6p21.3, encodes a proinflammatory cytokine involved in different biological activities (i.e., proliferation, differentiation, and death) and associated with the destruction of pancreatic  $\beta$ -cells (Duffy, 2007; Feng, et al., 2009). The cytotoxic T-lymphocyte associated protein 4 gene (*CTLA-4*), on chromosome

2q33, has been shown to be a negative regulator of T-cell activation during immune response (Kosmaczewska et al., 2001; Karman et al., 2012). The protein tyrosine phosphatase, non-receptor type 22 (lymphoid) gene (*PTPN22*), on chromosome 1p13.2, encodes the lymphoid-specific phosphatase (Lyp) and is involved in the prevention of spontaneous T-cell activation (Vang et al., 2007; Burn et al., 2011). *CTLA-4* and *PTPN22* deficiencies might therefore induce the proliferation of auto-reactive lymphocytes in autoimmune-mediated diabetes (Kosmaczewska et al., 2001; Burn et al., 2011).

The present study aimed to analyze the genetic association of three single nucleotide polymorphisms (SNPs) in the *CTLA-4* (rs231775), *PTPN22* (rs2476601), and *TNF- $\alpha$*  (rs1800629) genes with susceptibility to the development of T1D in Brazilian patients presenting with or without APSIII, as well as other autoimmune diseases, together or isolated.

## MATERIAL AND METHODS

### Subjects

We enrolled 205 patients with T1D diagnosed according to the clinical criteria established by the American Diabetes Association (2012). The mean age was 13.22 years (standard deviation (SD)  $\pm$  4.87) with 107/205 (52%) females and the average age at T1D onset was 7.33 years (SD  $\pm$  4.07). All patients were followed up at one of the three major pediatric endocrinology centers of the Public Health Service of Recife, Brazil (Instituto de Medicina Integral Professor Fernando Figueira - IMIP, Hospital da Restauração, or Hospital das Clínicas - UFPE).

In the control group, we enrolled 308 healthy individuals with no clinical evidence or family history of autoimmune diseases. The mean age was 27.5 years (SD  $\pm$  11.23), with 215/308 (70%) females. Healthy individuals were selected from the same geographical region as the patient group.

A free and informed consent was obtained from both patients and controls as well as from each person responsible for the patient and healthy individuals. The ethics committee from IMIP approved the study (IMIP No. 1717/2010).

### Diagnosis of autoimmune polyglandular syndrome

Four milliliter of peripheral blood samples collected in order to extract DNA and diagnose the presence of antibodies against thyroperoxidase (Anti-TPO) and transglutaminase (anti-tTg). Plasma samples (approximately 2 mL) were isolated from the whole blood stored at  $-80^{\circ}\text{C}$  and the rest of the sample proceeded to extraction and posterior storage at  $-20^{\circ}\text{C}$ .

For Anti-TPO, were performed a chemi-luminescence assay (Immulite anti-TPO Ab assay kit, Diagnostic products Co, Los Angeles, CA, USA). Patients with positive anti-TPO (titer exceeding 35 IU/mL, accordingly to the manufacturer suggestion) were considered as having AITD.

The presence of anti-tTg was determined by using an ELISA Eu-tTG kit (Eurospital, Trieste, Italy) following manufacturer instructions. Patients presenting with 10 AU (absorbance units) for anti-tTg antibodies were considered positive for CD.

The frequency of AITD in the patients with T1D was 21.4% (44/205); the percentage of patients with CD was 6.3% (13/205); patients with T1D characterized by both AITD and CD were 2.4% (5/205). The frequency of patients with T1D only was 69.9% (143/205).

### DNA extraction and single nucleotide polymorphism (SNP) genotyping

Genomic DNA was extracted from using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA), according to standard laboratory protocols. DNA quality and quantity was evaluated using Nanodrop spectrophotometer model 2000c (Thermoscientific, Waltham, MA, USA).

Three SNPs were selected for analysis, one per candidate gene. For *TNF- $\alpha$*  we selected a SNP located in the promoter region at position -308G/A (rs1800629); for *CTLA4* we analyzed the +49A/G polymorphism (rs231775) situated at exon 1; and for *PTPN22*, the non-synonymous SNP at the position +1858G/A (rs2476601) in exon 14 was considered.

All SNPs were genotyped using fluorescent allele-specific probes (TaqMan<sup>®</sup>, Life Technologies, Carlsbad, CA, USA) with TaqMan<sup>®</sup> Universal PCR Master Mix and using the ABI-7500 Real-Time PCR platform following the standard PCR protocol (Life Technologies). The TaqMan<sup>®</sup> assays and its respective SNP identifications are: C\_\_7514879\_10 for rs1800629; C\_\_2415786\_20 for rs231775; and C\_\_16021387\_20 for rs2476601.

### Statistical analysis

Allele and genotype frequencies of *CTLA4*, *TNF- $\alpha$* , and *PTPN22* SNPs were obtained by direct counting. The chi-square test with Yates' continuity correction was used to determine the association between the SNP distribution and the susceptibility to T1D and to clinical features as well as to evaluate conformation with Hardy-Weinberg equilibrium. All tests were performed using R software ([www.cran.us.rproject.org](http://www.cran.us.rproject.org)) through the SNPassoc package for R (R 2012). P values < 0.05 were considered statistically significant. However, for tests of gene interaction after the application of Bonferroni correction, P values < 0.016 were considered statistically significant.

## RESULTS

### Association of CTLA4, PTPN22, and TNF- $\alpha$ SNPs and susceptibility to T1D

The distributions of *CTLA4* (rs231775), *PTPN22* (rs2476601), and *TNF- $\alpha$*  (rs1800629) SNPs are reported in Table 1. The genotype frequencies in all studied groups were in Hardy-Weinberg equilibrium. The results of the association tests for the three SNPs are shown in Table 2.

The *PTPN22* +1858A allele (rs2476601) was significantly more frequent in patients with T1D (8%) than in healthy subjects (3%) (P = 0.0018; odds ratio (OR) = 2.52). Using the dominant model, the +1858 G/A+A/A genotypes were also statistically more frequent in patients with T1D+ (15%) than in healthy subjects (6%) (P = 0.0023; OR= 2.57).

Using the allelic model, a similar result was observed when comparing patients presenting only T1D vs HC (P = 0.0055; OR = 2.48) and patients with APSIII vs HC individuals (P = 0.0256; OR = 2.61); We also verified an association for the *PTPN22* +1858A allele by the dominant model with a P value = 0.0083 and OR = 2.48 for the T1D only vs HC comparison and P = 0.0225 (OR = 2.77) for the APSIII vs HC comparison.

No association was found in any comparison or model for the *CTLA-4* rs231775 (A/G) SNP in the *CTLA4* gene. We also did not observe an association between the *TNF- $\alpha$*  rs1800629 (G/A) SNP and the development of T1D, even when comparing patients presenting only T1D vs HC. However, when comparing individuals with APSIII with the T1D only group, we observed an association of this SNP in the allelic (P = 0.0442; OR = 0.44) and dominant models (P = 0.0387; OR = 0.40).

### Age-at-diagnosis and gene-gene interactions

**Table 1.** *TNF- $\alpha$*  (rs1800629), *CTLA4* (rs231775), and *PTPN22* (rs2476601) genotype and allele frequencies of healthy individuals and patients with type 1 diabetes mellitus stratified according to the insurgence of autoimmune polyglandular syndrome type III (APSIII).

	HC		T1D		T1D only		APSIII	
	N	Frequency	N	Frequency	N	Frequency	N	Frequency
<i>TNF-<math>\alpha</math></i> rs1800629								
G	532	0.89	358	0.87	243	0.85	115	0.93
A	68	0.11	52	0.13	43	0.15	9	0.07
GG	235	0.78	158	0.77	104	0.73	54	0.87
GA	62	0.21	42	0.20	35	0.24	7	0.11
AA	3	0.01	5	0.02	4	0.03	1	0.02
<i>CTLA-4</i> rs231775								
A	394	0.65	255	0.63	179	0.63	76	0.61
G	216	0.35	153	0.38	105	0.37	48	0.39
AA	127	0.42	82	0.40	59	0.42	23	0.37
AG	140	0.46	91	0.45	61	0.43	30	0.48
GG	38	0.12	31	0.15	22	0.15	9	0.15
<i>PTPN22</i> rs2476601								
G	596	0.97	378	0.92	264	0.92	114	0.92
A	20	0.03	32	0.08	22	0.08	10	0.08
GG	288	0.94	174	0.85	122	0.85	52	0.84
GA	20	0.06	30	0.15	20	0.14	10	0.16
AA	0	0.00	1	0.00	1	0.01	0	0.00

HC = healthy controls; T1D = type 1 diabetes; Freq. = frequency.

**Table 2.** Association between the three polymorphisms studied and type 1 diabetes (T1D) with or without autoimmune polyglandular syndrome (APSIII or T1D only) versus healthy individuals (HC).

Comparison	OR	95%CI	P value	OR	95%CI	P value	OR	95%CI	P value	OR	95%CI	P value
<i>TNF-<math>\alpha</math></i> (rs1800629)												
		G vs A			GG vs GA			GG vs GA			GG vs GA+AA	
HC vs T1D	1.14	(0.77-1.67)	0.5810	1.01	(0.65-1.57)	0.9371	2.48	(0.58-10.52)	0.3641	1.08	(0.70-1.65)	0.8215
HC vs T1D only	1.38	(0.92-2.09)	0.1477	1.28	(0.79-2.05)	0.3770	3.01	(0.66-13.70)	0.2785	1.36	(0.86-2.15)	0.2373
HC vs T1D+APSIII	0.61	(0.30-1.26)	0.2380	0.49	(0.21-1.13)	0.1292	1.45	(0.15-14.22)	0.7464	0.54	(0.24-1.18)	0.1640
T1D only vs APSIII	0.44	(0.21-0.94)	0.0442	0.39	(0.16-0.92)	0.0453	0.48	(0.05-4.41)	0.8573	0.40	(0.17-0.90)	0.0387
<i>CTLA4</i> (rs231775)												
		A vs G			AA vs AG			AA vs GG			AA vs AG+GG	
HC vs T1D	1.09	(0.84-1.42)	0.5397	1.01	(0.61-1.44)	0.9494	1.26	(0.73-2.19)	0.4880	1.06	(0.74-1.52)	0.8162
HC vs T1D only	1.07	(0.80-1.43)	0.7051	0.94	(0.61-1.44)	0.8559	1.25	(0.68-2.29)	0.5817	1.00	(0.67-1.50)	0.9322
HC vs T1D+APSIII	1.15	(0.77-1.71)	0.5516	1.18	(0.65-2.14)	0.6855	1.31	(0.56-3.06)	0.6949	1.21	(0.69-2.13)	0.6019
T1D only vs APSIII	1.08	(0.70-1.66)	0.8241	1.26	(0.66-2.42)	0.5923	1.05	(0.42-2.61)	0.8962	1.21	(0.65-2.23)	0.6590
<i>PTPN22</i> (rs2476601)												
		G vs A			GG vs GA			GG vs GA			GG vs GA+AA	
HC vs T1D	2.52	(1.42-4.48)	0.0018	2.48	(1.23-4.54)	0.0036	ND	ND	0.8011	2.57	(1.42-4.64)	0.0023
HC vs T1D only	2.48	(1.33-4.63)	0.0055	2.36	(1.23-4.54)	0.0142	ND	ND	0.6608	2.48	(1.30-4.74)	0.0083
HC vs T1D+APSIII	2.61	(1.19-5.73)	0.0256	2.77	(1.23-6.25)	0.0225	ND	ND	ND	2.77	(1.23-6.25)	0.0225
T1D only vs APSIII	1.05	(0.48-2.29)	0.9431	1.17	(0.51-2.68)	0.8695	0	ND	0.6562	1.12	(0.49-2.54)	0.9579

The results for the allelic, co-dominant and dominant models are shown from left to right, respectively. OR = odds ratio; CI = confidence intervals.

Age-at-diagnosis of T1D and gene-gene interaction associations among rs1800629, rs231775, and rs2476601 SNPs were also evaluated. No association with gene-gene interaction and T1D was found for any loci tested ( $P > 0.05$ ). However, the mean age-at-diagnosis was significantly different according to the genotypes of *TNF- $\alpha$*  and *PTPN22*. In the over-dominant model the mean ages-at-diagnosis were 7.24 and 8.95 for the *TNF- $\alpha$*  G/G+A/A vs G/A genotypes, respectively ( $P = 0.014$ ). The mean ages-at-diagnosis for the G/G and G/A+A/A in the dominant model for the *PTNP22* SNP were 7.87 and 6.10, respectively ( $P = 0.025$ ). However, after Bonferroni

correction, these associations were no longer statistically significant.

## DISCUSSION

T1D is a multifactorial autoimmune disorder with an HLA-specific main locus that has been shown to be responsible for 40% of the susceptibility to T1D. However, previous genome wide association studies have demonstrated associations of other loci from non-HLA regions with T1D onset susceptibility. These non-HLA loci might modulate and modify the course of disease, i.e., disease progression, clinical manifestation, and the onset of other autoimmune disorders. In this study we analyzed three SNPs in three non-HLA classical loci previously implicated in the susceptibility to develop T1D or other associated autoimmune diseases: *CTLA-4* (rs231775), *PTPN22* (rs2476601), and *TNF- $\alpha$*  (rs1800629).

Feng et al. (2009) reported in their meta-analysis that the association of the polymorphism rs1800629, which represents a G>A transition at the -308 position in *TNF- $\alpha$* , with T1D is primarily found in Asian populations. The -308A allele might increase *TNF- $\alpha$*  protein production *in vitro* (Lee et al., 2005) and could be associated with the onset of T1D. In our study, we did not observe any association of this SNP with T1D development. We are aware that *TNF- $\alpha$*  and the rs1800629 genetic variant considered in this study are in strong linkage disequilibrium with the class II HLA region, although it is not clear whether *TNF- $\alpha$*  polymorphisms at the promoter region have an independent role in the predisposition to T1D or if they show association through a “hitchhiking effect” (Deja et al., 2006; Feng et al., 2009). However, we observed a marginal association between the *TNF- $\alpha$*  -308 variant and the development of APSIII, as compared to individuals exhibiting only T1D. This finding suggests that this cytokine might be involved in the common pathways underlying the development of multiple autoimmune diseases. Fourati et al. (2012) found an association of the rs1800629 SNP with APSIII in Tunisian patients, although they compared APSIII individuals with healthy controls. Therefore, as the published results for *TNF- $\alpha$*  are weak and controversial, more replica studies are needed.

The genetic associations between the *CTLA4* polymorphic rs231775 variant have been previously investigated in different ethnic groups, but with inconsistent findings (Si et al., 2012; Chen et al., 2013). In a meta-analysis, Chen et al. (2013) evaluated 52 studies and concluded that a modest association between the +49A>G polymorphism with T1D risk was indicated, with a related ethnic component. However, in a case-control study performed in a cohort of Turkish children with T1D, no association was observed between this polymorphism and increased susceptibility to T1D or with the clinical and laboratory characteristics of the patients with T1D ( $P > 0.05$ ) (Çelmeleli et al., 2013). Furthermore, in another study by Rodríguez et al. (2014), no significant association was found for *CTLA4* in the development of T1D in a Colombian population. These results are in agreement with those found in our population.

We also found no association for the +49 A>G polymorphism in the APSIII comparisons. However, Villano et al. (2009) observed an association of this SNP with APSIII when considering only individuals with simultaneous T1D and AITD, although these results have not been replicated in a Japanese cohort (Horie et al., 2012). In contrast, in our APSIII group we enrolled not only individuals with T1D and AITD, but also individuals with T1D and CD and even patients with simultaneous T1D, AITD and CD as well.

The 1858A+ variant of the *PTPN22* gene, also known as R620W, was associated with T1D in our study population from Brazil. In fact, the +1858A allele was more frequent in T1D+ patients

(OR = 2.52; CI=1.42-4.48) than in healthy subjects. These findings are in agreement with the meta-analysis performed by Lee et al. (2007), wherein the authors demonstrated that the +1858A allele conferred susceptibility to RA, SLE, Graves' disease (GD), as well as T1D, supporting evidence of an association of the *PTPN22* gene with subgroups of autoimmune diseases. Furthermore, in a recent study the same authors confirmed that the rs2476601 *PTPN22* polymorphism was associated with T1D susceptibility in Europeans (Lee and Song, 2012). For a Colombian population, as well as in our study, an association was found for the *PTPN22* gene and development of T1D (Rodríguez et al., 2014). Tang et al. (2012) indicated that T1D is associated with the *PTPN22* +1858G/A gene polymorphism, and that association with this promoter polymorphism was likely dependent on ethnicity.

In our study, we also described an association of the *PTPN22* +1858G/A variant and APSIII onset, when compared with HC individuals. Similar studies from Villano et al. (2009) and Dultz et al. (2009) also found significant results.

Overall, such positive results indicate that an individual carrying these alleles is at risk to develop both T1D and AITD, and furthermore, they suggest that T1D and CD might also share similar pathways and pathogenic mechanisms.

No association with age-at-diagnosis and gene-gene interaction between the three SNPs and T1D was observed in the Brazilian population in our study. The reports of age-at-diagnosis interaction effects at non-HLA loci (Howson et al., 2012) as well as the study of gene-gene interactions of T1D-associated regions are contradictory (Payne et al., 2007), supplementary studies focusing on these fields should be performed.

In conclusion, our study, even with the limitation of a small number of patients analyzed (which numbers are even lower when considering individuals with the combined diagnoses of T1D, AITD, and CD), suggests an association between a *CTLA4* SNP (rs231775) and the susceptibility to develop T1D and other autoimmune diseases in Brazilian patients. However, divergent results of association between the *CTLA4* gene and T1D have been found across various studies: these apparent discrepancies might be attributed to several factors, including differences in genetic background (Marron et al., 1997; Ikegami et al., 2006), possible linkage to HLA susceptibility haplotypes, and patient selection (Ikegami et al., 2006).

Unlike this study, due to the higher prevalences, most previous studies only investigated potential associations of *PTPN22*, *CTLA4*, and *TNF- $\alpha$*  with T1D, AITD, and CD as individual diseases. Our findings provide new insights into the genetic components associated with the susceptibility to T1D in Brazilian patients, and reinforce the importance of discriminating whether a patient presents with other autoimmune diseases that might be correlated with T1D.

## Conflicts of interest

The authors declare no conflicts of interest.

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