Zinc transporter 8 autoantibodies in patients with type 1 diabetes from a multiethnic population and their first degree relatives

Autoanticorpos transportadores de zinco 8 em pacientes com diabetes tipo 1 de uma população multiétnica e seus parentes de primeiro grau

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ABSTRACT

Objective: Zinc transporter 8 autoantibodies (ZnT8A) have been poorly studied in non-Caucasian individuals. We aimed to investigate the prevalence of ZnT8 autoantibodies in patients with T1D and their first degree relatives (FDR) from a multiethnic population, as well as its relation with the insulin (INS) or the protein tyrosine phosphatase non-receptor 22 (PTPN22) gene polymorphisms. **Subjects and methods:** ZnT8A were analyzed in sera from T1D patients (n = 72, mean age of 30.3 ± 11.4 years) of variable duration (15.7 ± 11.8 years) and their FDR (n = 78, mean age of 18.3 ± 9.1 years) by a triple mix Radioligand Binding Assay (RBA) for the ZnT8 autoantibody (ZnT8-RWQ) variants. SNP (single nucleotide polymorphism) for INS and PTPN22 were genotyped. **Results:** The prevalence of ZnT8A was higher in T1D patients than FDR, for ZnT8TripleA (24% *vs.* 4%, p = 0.001), ZnT8RA (24% *vs.* 4%, p < 0.001) and ZnT8QA (15% *vs.* 3%, p = 0.004). All FDR with ZnT8A (n = 3) had at least another positive antibody. Heterozygosis for PTPN22 was associated with a higher frequency of ZnT8TripleA (p = 0.039) and ZnT8RA (p = 0.038). **Conclusions:** ZnT8A is observed in non-Caucasian patients with T1D, even years after the disease onset, as well as in their FDR. In those, there was an overlap between ZnT8A and other T1D antibodies. ZnT8A was associated with PTPN22 polymorphisms. Further longitudinal studies are necessary to elucidate the importance of these findings in the natural history of T1D patients with multiethnic background. Arq Bras Endocrinol Metab. 2014;58(7):737-43

Keywords
Autoimmunity; diabetes; non-whites; ZnT8A; PTPN22

RESUMO

Objetivo: Os autoanticorpos transportadores de zinco 8 (ZnT8A) foram pouco estudados em indivíduos não caucasianos. Nosso objetivo foi investigar a prevalência de autoanticorpos ZnT8 em pacientes com T1D e seus parentes de primeiro grau (PPG) em uma população multiétnica, assim como a sua relação com os polimorfismos genéticos da insulina (INS) ou proteína tirosina fosfatase não receptora tipo 22 (PTPN22). **Sujeitos e métodos:** ZnT8A foram analisados no soro de pacientes com T1D (n = 72, idade média de 30,3 ± 11,4 anos) de duração variável (15,7 ± 11,8 anos) e seus PPG (n = 72, idade média de 30,3 ± 11,4 anos) usando-se um ensaio de competição com radioligantes (RBA) para variantes dos autoanticorpos ZnT8 (ZnT8-RWQ). Os polimorfismos de nucleotídeo único para a INS e PTPN22 foram genotipados. **Resultados:** A prevalência de ZnT8A foi mais alta em pacientes T1D do que nos PPG, para ZnT8TriploA (24% contra 4%, p = 0,001), ZnT8RA (24% contra 4%, p < 0,001) e ZnT8QA (15% contra 3%, p = 0,004). Todos os PPG com ZnT8A (n = 3) apresentaram positividade para pelo menos outro anticorpo. A heterozigose para PTPN22 foi associada a uma frequência mais alta de ZnT8TriploA (p = 0,039) e de ZnT8RA (p = 0,038). **Conclusões:** Os ZnT8A foram observados em pacientes não caucasianos com T1D, mesmo depois de anos do início da doença, assim como em seus PPG. Nos parentes, houve uma sobreposição entre os ZnT8A e outros anticorpos para T1D. Os ZnT8A mostraram-se associados aos polimorfismos PTPN22. São necessários outros estudos longitudinais para se elucidar a importância desses achados na história natural de pacientes com T1D com antecedentes étnicos variados. Arq Bras Endocrinol Metab. 2014;58(7):737-43

Descritores
Autoimunidade; diabetes; não brancos; ZnT8A; PTPN22

INTRODUCTION

Type 1 diabetes (T1D) is a chronic disease characterized by an autoimmune destruction of the pancreatic islet β cells (1,2). The immunogenetic and environmental factors associated with the initiation and progression of T1D are not yet fully understood. However, elucidation of these issues is crucial for possible prevention and treatment of T1D. Most investigations regarding the course of T1D in humans have included only Caucasians, therefore very little is known about the natural history of T1D in other ethnic groups.

Autoantibodies against insulin, glutamic acid decarboxylase 65 (GAD65) and islet antigen-2 (IA2) are important predictive markers preceding clinical onset of T1D (2,3). Zinc Transporter 8 (ZnT8) protein was recognized as one of the four major autoantigens in T1D patients (4) and to which autoantibodies are generated prior to the clinical onset of T1D. ZnT8 autoantibodies (ZnT8A) have been demonstrated to substantially overlap with the prevalence of IA2A, in particular (5). Moreover, a significant correlation of the prevalence of ZnT8A and IA2A has been proven in T1D patients (6).

ZnT8A have been suggested to be directed against at least two epitopes found in relation to the single amino acid in position 325 expressed on the C-terminal portion of the protein, and less frequently against the N-terminal portion (4,5). The description of the single nucleotide polymorphism (SNP) rs13266634 in the gene of ZnT8, *SLC30A8*, is explaining an amino acid change in position 325 from arginine (CGG) to tryptophan (TGG) (7). In addition, the SNP rs16889462 at the same amino acid position in *SLC30A8* encodes glutamine (CAG). To date, there are three variants of ZnT8A detected in T1D patients, ZnT8RA (arginine), ZnT8WA (tryptophan) and ZnT8QA (glutamine) (8-10). The autoantibody specificity of ZnT8RA and ZnT8WA, respectively, has been shown to be determined by the rs13266634 genotype (5,6). In addition, the ZnT8RA variant was associated with the high risk HLA-DQB1*0302 genotypes (6).

The amino acid residues of ZnT8 are not equally prevalent in populations although the ZnT8RA variant is most common with a prevalence of 75% in European Caucasians, 98% in African Americans, and 50% in Asians (11). ZnT8W is prevalent in 25% of Europeans, 2% of African Americans, and in nearly 50% of Asians compared to the ZnT8Q variant, which is rarely found in Europeans and Asian but in 9% of the African American population (11).

The protein tyrosine phosphatase, non-receptor type 22 (PTPN22) gene encodes a protein tyrosine phos-

phatase expressed in the lymphoid (LYP) (12,13). The importance of the PTPN22 gene and the mechanisms of SNP R620W (rs2476601) are not fully understood, although it has previously been evident as genetic predisposition risk marker for autoimmune diseases, such as T1D and rheumatoid arthritis (12-14). It has been postulated that carriers of the PTPN22 SNP R620W may have a disrupted suppression of T-cell activation (15), resulting in an overactive T-cell response (16).

The aim of this study was to evaluate the prevalence of ZnT8 autoantibodies, as well as its association with insulin (INS) gene and protein tyrosine phosphatase non-receptor 22 (PTPN22) gene polymorphisms in multiethnic Brazilian patients with TID and their first degree relatives (FDR).

SUBJECTS AND METHODS

Subjects

Brazilian patients with T1D (n = 72) and their FDR (n = 78) were interviewed and blood was sampled for DNA extraction and autoantibody measurement. T1D was defined according to the American Diabetes Association criteria. One participant per family was included. Participants were classified as whites and non-whites (mostly Afro-descendants) based on their phenotype and family background. Although white individuals in this population have predominantly Caucasian ancestry, they could not be classified as pure Caucasians due to miscegenation in past generations (17). The project was approved by the institutional review board and all participants signed an informed consent.

Serum samples were analyzed by a standard radioligand binding assay (RBA) for the three individual ZnT8A variants (ZnT8RA, ZnT8WA, ZnT8QA) as well as with the ZnT8TripleA assay, which both were developed at the Department of Clinical Sciences, Skäne University Hospital, Malmö, Sweden. The RBA for GAD, insulin and tyrosine phosphatase A (GADA, IAA and IA2A, respectively) were performed at the Federal University of Rio de Janeiro, Rio de Janeiro, Brazil.

Generation of ZnT8R, ZnT8W, ZnT8Q constructs

The C-terminal cDNA was constructed with arginine at amino acid 325 from human islet ZnT8 (SLC30A8) (gift from Dr. J. C. Hutton at Barbara Davis Center for Childhood Diabetes, University of Colorado at Denver and Health Sciences Center, Aurora, CO). The gene-

ration of the ZnT8 constructs is described in detail by Vaziri-Sani and cols. (18). Briefly, the ZnT8R construct of amino acid (aa) 268-369 was subcloned into the pTnT™ vector (Promega, Madison, USA) and was further used as a template in a Phusion™ site-directed mutagenesis kit (Finnzymes Oy, Espoo, Finland) to generate additional plasmids, pThZnT8W and pThZn-T8Q (6,9,10).

Radioligand binding assay (RBA)

The RBA detection of all three ZnT8A variants was carried out as described elsewhere (18). Briefly, the recombinant ^{35}S-methionine labeled ZnT8 protein variants of cytosolic segments (aa 268-369) were produced using the coupled *in vitro* transcription translation system (Promega, Madison, WI). Sera (5 μL) were incubated with 60 μL labeled ZnT8 autoantigen overnight at 4°C at 300 rpm and separated by precipitation using Protein A Sepharose (Invitrogen, Carlsbad, CA). Antibody-bound radioactivity was counted in a β-counter (1450 MicroBeta TriLux Microplate Scintillation-Luminescence Counter) and converted into the in-house units (U) using individual standard curves generated by six step doubling dilutions of high-titer T1D sera with high reactivity for each individual ZnT8 autoantigen. For the ZnT8ATriple assay, sera were incubated with a mixture of all three labeled ZnT8 variants (ZnT8R, ZnT8W and ZnT8Q) at equal concentrations. Cut-off values for the positive test were set to 75 U/mL for ZnT8RA and ZnT8WA, 100 U/mL for ZnT8QA and 60 U/mL for the ZnT8ATriple assay. Cut-off values were based on the 98th percentile observed in 398 healthy adult controls from Malmö, Sweden.

Titers of the three ZnT8A variants and ZnT8TripleA were measured for both T1D patients and their FDR. The titers of GADA, IAA and IA2A were measured only in samples from FDR.

Genetic analysis

The T1D patients and their FDR were genotyped for INS gene and PTPN22 polymorphisms. SNP for INS and PTPN22 (polymorphism RS620W rs2476601) were genotyped with fluorogenic allele-discrimination chemistry as described elsewhere (14,16,19).

Statistical analysis

Mann-Whitney U test and chi-square were used for comparison between groups. Spearman coefficient was used to test correlation between continuous variables, and Fisher's exact t-test to test correlation between categorical variables. A p-value < 0.05 was considered significant.

RESULTS

Epidemiological and genetic characteristics of the study group

The characteristics of the study group are described in table 1. T1D subjects were older (mean ± SD years, 30.3 ± 11.4) than the FDR (mean ± SD years, 18.3 ± 9.1) participating in the study. The median age of T1D onset was 18.6 ± 11.6 years and its duration varied from 1 to 30.5 years (mean ± SD years, 15.7 ± 11.8).

ZnT8A profile in the T1D patients and FDR

The prevalence of ZnT8As was higher in T1D patients than in FDR, for ZnT8TripleA (24% *vs.* 4%, p = 0.001), ZnT8RA (24% *vs.* 4%, p < 0.001) and ZnT8QA (15% x 3%, p = 0.004). For ZnT8WA no difference was found (8.3% *vs.* 2.6%; p = 0.154) between groups (Table 1). ZnT8RA were most prevalent in both T1D patients and FDR (Table 1). Among the T1D patients, 5.5% (n = 4) had ZnT8RA alone (Figure 1) compared to 1.3% (n = 1) in the FDR (Figure 2), and 11% (n = 8) had autoantibodies against all three ZnT8A variants compared to 2.6% (n = 2) in the FDR. In those with positive antibodies, the titer levels of ZnT8ATriple (p = 0.030), ZnT8RA (p = 0.006) and ZnT8WA (p = 0.01) were also higher in patients compared to the FDR. This was not true for the ZnT8QA titers. ZnT8A positivity or its titers were not associated with ethnicity in either groups (Positivity: p = 0.783 for patients and p = 0.557 for FDR; titers 117.1 U/ml ± 369.2 for patients and 71.1 U/ml ± 426.3 for FDR) and did not show any association with the presence of diabetic ketoacidosis at diagnosis (p = 0.574).

There were no differences in the frequency nor the titers of any of the autoantibody variants (p = 0.507 for ZnT8ATriple, p = 0.507 for ZnT8RA, p = 0.119 for ZnT8WA and p = 0.112 for ZnT8QA) between patients who had diabetes for less than five years (n = 16) compared to those with a longer disease duration (n = 56). However, for ZnT8QA there was a trend towards higher titers in the former group than the latter (p = 0.056; 204.68 U/mL ± 523.95 *vs.* 39.64 U/mL ± 65.45). ZnT8A positivity was seen in a patient with 30.5 years of disease.

Table 1. Epidemiological and genetic characteristics of type 1 diabetes patients and their first degree relatives (FDR)

Characteristics	T1D patients n = 72 (%)	FDR n = 78 (%)	*p* value
Age (mean ± SD years)	30.3 ± 11.4	18.3 ± 9.1	< 0.001
Age onset (mean ± SD years)	18.6 ± 11.6	–	–
Duration of T1D (mean ± SD years)	15.75 ± 11.8	–	–
Gender n (%) Female	49 (68)	45 (57.7)	0.419
Ethnicity n (%)			
Whites	31 (43)	31 (39.7)	0.751
Non-whites	41 (57)	47 (60.3)	
INS gene SNP n (%)			
Homozygous T/T	38 (55.9)	29 (40.8)	0.201
Heterozygous A/T	21 (30.9)	32 (45.1)	
Polymorphic allele A	9 (13.2)	10 (14.1)	
Total	68 (100)	71 (100)	
PTPN22 gene SNP R620W rs2476601 (%)			
Homozygous C/C	56 (84.8)	56 (93.3)	0.438
Heterozygous C/T	10 (15.2)	4 (6.7)	
Homozygous T/T	0 (0)	0 (0)	
Total	66 (100)	60 (100)	
ZnT8A frequency (%)			
TripleA	17 (23.6)	3 (3.8)	0.001
ZnT8RA	17 (23.6)	3 (3.8)	< 0.001
ZnT8WA	10 (13.9)	2 (2.6)	0.154
ZnT8QA	6 (8.3)	2 (2.6)	0.004
Total	72 (100)	78 (100)	
ZnT8A titers (U/ml) (mean ± SD) (median)			
TripleA	117.1 ± 369.2; 15,00	71.1 ± 426.3; 11,00	0.030
ZnT8RA	173.5 ± 454.4; 18,00	97.1 ± 491.3; 15,50	0.006
ZnT8WA	59.7 ± 267.9; 21,00	45.5 ± 294.4; 14,00	0.010
ZnT8QA	76.3 ± 257.0; 12,00	48.3 ± 251.5; 9,00	0.095

72 T1D subjects and 78 FDR subjects were analyzed for ZnT8ATriple, ZnT8RA, ZnT8WA and ZnT8QA. T1D patients (n = 68) and FDR (n = 71) were analyzed for the INS gene SNP. T1D patients (n = 66) and FDR (n = 60) were analyzed for PTPN22 gene SNP R620W (rs2476601).

T1D: type 1 diabetes; INS: insulin; PTPN22: protein tyrosine phosphatase non-receptor 22; ZnT8A: Zinc transporter 8 autoantibody.

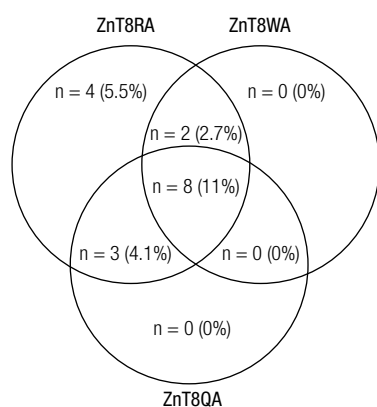P-value < 0.05 was considered significant.

Figure 1. The prevalence of Zinc transporter 8 autoantibodies (ZnT8A) against ZnT8-Arginine (R), ZnT8-Tryptophane (W) and ZnT8-Glutamine (Q) in type 1 diabetes patients (T1D) (n = 72) analyzed by an individual Radioligand binding assay (RBA). Among the T1D patients 11% (n = 8) had all three ZnT8 autoantibodies.
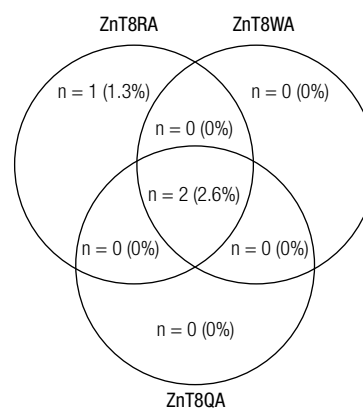

Figure 2. The prevalence of ZnT8 autoantibody (ZnT8A) variants, ZnT8-Arginine (R), ZnT8-Tryptophane (W) and ZnT8-Glutamine (Q) in first degree relatives (FDR) (n = 78) to T1D patients analyzed by an individual Radioligand binding assay (RBA). Among the FDR 2.6% were positive for all three ZnT8A variants.
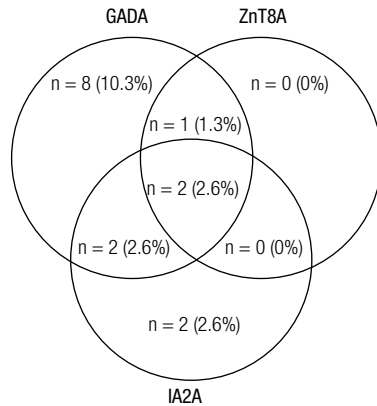
Figure 3. Prevalence of autoantibodies against Glutamic acid decarboxylase (GADA), Zinc transporter 8 (ZnT8) (any of the variants) and insulinoma-associated protein 2 (IA2) analyzed in first degree relatives (FDR) (n = 78) to T1D patients. GADA were most prominent (10.3%) of the three GADA, ZnT8A and IA2A autoantibody types, while all of them were detected in 2.6% of the FDR.

Overlap of ZnT8A prevalence with other islet autoantibodies in FDR

GADA, IA2A and IAA were tested in FDR of patients with T1D. One or more autoantibodies (GADA, IA2A and one, two or all variants of ZnT8A) were found in 15.6% of the FDR. GADA was detected in 10% (n = 8) and IA2A in 2.6% (n = 2). These results are shown in figure 3. In antibody positive (Ab+) FDR, the mean titers of GADA and IA2A were 23.9 U/mL (SD 25.1 U/mL) and 5.8 U/mL (SD 5.4 U/mL), respectively. Among Ab+ FDR, we found a positive association between GADA and ZnT8TripleA (p = 0.001) as well as with all individual variants, ZnT8RA (p = 0.001), ZnT8WA (p = 0.002) and ZnT8QA (p = 0.012). Similar overlap was seen for IA2A with the ZnT8TripleA (p = 0.007), ZnT8RA (p = 0.006), ZnT8WA (p = 0.002) and ZnT8QA (p = 0.012). Only one FDR was positive for IAA and no association was found with the three ZnT8A variants. The number of Ab+ individuals did not increase with the inclusion of ZnT8A measurement.

Association between ZnT8A and the PTPN22 polymorphism

The genotype distribution of the INS gene (n = 139, T/T 48.2%, A/T 38.1%, A/A 13.7%) and the PTPN22 (n = 126, C/C 88.9%, C/T 11.1%, T/T 0%) including both patients and FDR were heterogeneous (p = 0.201 for INS, p = 0.438 for PTPN22) (Table 1). Interestingly, we found an association between PTPN22 genotype and the prevalence of ZnT8 antibo-

dies. Heterozygotes for PTPN22 had a higher frequency of ZnT8TripleA (35.7% *vs.* 12.61%; p = 0.039) and also with the individual ZnT8RA (35.7% *vs.* 12.5%; p = 0.038), but not of ZnT8WA (7.1% *vs.* 5.4%; p = 0.571) nor ZnT8QA (21.4% *vs.* 8.9%; p = 0.159). Moreover, heterozygotes for PTPN22 had higher titers of all ZnT8 antibodies (327.9 U/mL ± 778.9 *vs.* 79.6 ± 366.5 and p = 0.014 for ZnT8TripleA; 506.14 U/ml ± 1037.1 *vs.* 110.6 ± 393.1 and p = 0.002 for ZnT8RA; 191.28 U/mL ± 597.7 *vs.* 43.1 and p = 0.03 for ZnT8WA; 207.8 ± 563.3 *vs.* 51.2 ± 213.9 and p = 0.048 for ZnT8QA). However, when patients and FDRs were analyzed separately, the association between PTPN22 polymorphism and ZnT8A was not seen in either of the groups.

DISCUSSION

In this study, we investigated the prevalence of ZnT8 autoantibodies in patients with T1D and their FDR with multiethnic background, as well as its relation to the INS and PTPN22 gene polymorphisms. As the majority of studies in this field include white Caucasians or Asiatics, the immunogenetic profile of Afro-descendants remains unclear. Therefore, analysis of this group is especially interesting.

In our population, Znt8A prevalence was lower than previously reported in Caucasians FDR of patients with T1D (4% *vs.* 24%) (4,9,10), but similar to that reported in Asiatics (27). The most prevalent ZnT8A variant was the autoantibody directed against arginine, which is in concordance with Wenzlau and cols. (8). Among the FDR with positivity for ZnT8A, all had the variant arginine, suggesting that this epitope is the most important in our population. In FDR of patients with T1D with multiethnic background, the measurement of ZnT8A did not improve the stratification for T1D risk, as all individuals were positive for ZnT8A and GADA concomitantly. In FDR, there was an overlap not only of ZnT8A (all variants) and GADA, but also IA2A. Previous studies have reported similar findings at the onset of T1D in Caucasian patients (4,9,20,29) and FDR (26,30). Therefore, ZnT8A can be considered a marker for T1D autoimmunity not only in Caucasians, but also in multiethnic populations such ours, although it does not seem to improve the identification of FDR in risk of T1D when added to GADA, IA2A and IAA.

Interestingly, ZnT8A was detected among T1D patients even many years after diagnosis, which was previ-

ously shown by Yang and cols. in the Chinese population (27). However, Howson and cols. demonstrated a higher prevalence of ZnT8A in the first two years of the disease (31). The factors involved in the maintenance of serum ZnT8A are still to be identified, but this characteristic makes the measurement of this antibody particularly useful in patients with long-standing diabetes and unclear diabetes classification, where the identification of an autoimmune marker can establish the definite diagnosis. Moreover, Trabucchi and cols. have shown that a significant proportion of autoimmune adult-onset diabetic patients present ZnT8A as the only humoral marker (32).

A major finding in our study was the association between PTPN22 and ZnT8RA in a multiethnic population, which differ from previous data (31,33). Although we did not find any correlation between PTPN22 genotype and ZnT8A in each group individually (patients or FDR), this may be due to the limited size sample. Other authors did not show a similar association. Sorgjerd and cols. identified a trend for an association between ZnT8A positivity and the polymorphisms of the SLC30A8 gene (28). For Howson and cols., only *FCRL3* on chromosome 1q23.1 and the HLA class I region were associated with positivity for ZnT8A (31). The association between SLC3A08 and ZnT8WA has also been reported by Delli and cols. in Swedish patients with T1D (29).

It is possible that there is a link between PTPN22 polymorphism and ZnT8A specifically in our population or other similar ethnic groups. PTPN22 SNP R620W is known to mediate the risk for several autoimmune diseases including T1D, and may therefore be important in the regulation of autoreactivity in general (12-14,16). PTPN22 has been linked to the presence of other antibodies linked to T1D such as GADA and IA2A, both in individuals at risk for T1D, as well as short or long-standing disease (16,22,24). However, we did not identify any association of PTPN22 and GADA or IA2A in our cohort of patients (25) and FDR (19) with T1D. To our knowledge, this is the first study to identify such correlation between the PTPN22 gene and ZnT8RA, which could be a peculiarity of our population. This association needs to be further investigated in order to elucidate how PTPN22 could be involved in the pathogenesis of T1D, which in turn could be useful in future prevention strategies.

In conclusion, ZnT8A is observed in non-Caucasian patients with T1D, even years after the disease onset, as well as in their FDR. In those, there was an overlap between ZnT8A and other T1D antibodies. ZnT8A was associated with PTPN22 polymorphisms. Further longitudinal studies are necessary to elucidate the importance of these findings in the natural history of T1D patients with multiethnic background.

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