Autoimmunity, 2011; Early Online: 1–6

ISSN 0891-6934 print/1607-842X online
DOI: 10.3109/08916934.2011.604658

informa
healthcare

Detection and characterization of ZnT8 autoantibodies could help to screen latent autoimmune diabetes in adult-onset patients with type 2 phenotype

ALDANA TRABUCCHI[1,2], NATALIA I. FACCINETTI[1,2], LUCIANO L. GUERRA[1,2], FÉLIX M. PUCHULU[3], GUSTAVO D. FRECHTEL[4], EDGARDO POSKUS[1,2], & SILVINA N. VALDEZ[1,2]

[1]*Chair of Immunology, School of Pharmacy and Biochemistry, University of Buenos Aires (UBA), Buenos Aires, Argentina,* [2]*Humoral Immunity Institute Prof. Ricardo A. Margni (IDEHU), National Research Council (CONICET)-UBA, Buenos Aires, Argentina,* [3]*Diabetes Unit, Clinical Hospital José de San Martín, Buenos Aires, Argentina, and* [4]*Chair of Genetics and Molecular Biology, School of Pharmacy and Biochemistry, UBA, Buenos Aires, Argentina*

(*Submitted 13 April 2011; revised 8 June 2011; accepted 8 July 2011*)

Abstract

Autoantibodies to zinc transporter 8 (ZnT8A) constitute an additional marker of autoimmune diabetes, complementing those already used in diagnosis support. ZnT8A could also be found in latent autoimmune diabetes of adults (LADA). The aim of this study was to evaluate the prevalence of ZnT8A in adult-onset diabetic patients in Argentinian population. A total of 271 patients diagnosed for diabetes at mean age 53.4 ± 10.9, body mass index ≤30, without insulin treatment for the first year of disease, and initially classified as type 2 diabetic patients were tested for ZnT8A using cDNA plasmids encoding the C-terminal domains (aa 268–369) carrying 325Arg, 325Trp, and a dimeric cDNA construct carrying both 325Arg and 325Trp (ZnT8 Arg–Trp325). We also analyzed proinsulin autoantibodies (PAA), glutamic acid decarboxylase autoantibodies (GADA), and protein tyrosine phosphatase IA-2 autoantibodies (IA-2A). A subset of 101 patients was followed during 6 years in order to analyze insulin requirement. Out of the 271 patients, 22.1% presented at least one humoral marker, 2.6% were PAA +, 12.5% were GADA +, 3.3% were IA-2A +, and 10.7% were ZnT8A +. Among the latter, 7.0% were ZnT8A–Arg325, 51.7% were ZnT8A–Trp325, and 62.1% were ZnT8A–Arg–Trp325. Furthermore, the prevalence of autoantibodies in the group of patients treated with insulin ($n = 18$) was 55.6%. These results demonstrated that a significant proportion of autoimmune adult-onset diabetic patients presented ZnT8A as the only humoral marker. Between them, the higher prevalence was for ZnT8A–Trp325. We suggest that screening for LADA patients, best performed with a minimal set of marker determination, must include at least the screening of GADA and ZnT8A–Arg–Trp325.

Keywords: *Zinc transporter 8, humoral immune response, adult-onset diabetes mellitus, diagnostic support, ZnT8A prevalence*

Introduction

Zinc transporters are multipass transmembrane proteins belonging to the SLC30 protein family with a role in the transport of zinc into the vesicles [1]. In the pancreas, insulin-secreting β cells accumulate very high amounts of zinc, which is mainly stored inside secretory vesicles forming a solid insulin hexamer [2,3]. In response to external stimuli, exocitosis of insulin occurs, insulin granules fuse with the β-cell plasma membrane and release insulin and zinc to circulation [4]. Zn^{2+} ions are also required for proinsulin (PI) aggregation and storage in the secretory pathway, and are an essential cofactor of carboxypeptidase E, which plays an important role in the conversion of PI to insulin. Thus, zinc is an important mediator of insulin storage and secretion, and β cells need efficient transporters to accumulate zinc in cytoplasmic vesicles [5]. In particular, zinc transporter 8 (ZnT8) is a pancreatic β-cell secretory granule membrane protein that has been recently identified as a target humoral immunity in type 1 diabetes [6,7].

Correspondence: S. N. Valdez, Cátedra de Inmunología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 4to piso (C1113AAD), Buenos Aires, Argentina. Tel: 54 114964 8260. Fax: 54 114964 0024. E-mail: silval@ffyb.uba.ar

Autoantibodies to ZnT8 (ZnT8A) constitute an additional marker of autoimmune diabetes, complementing those already used in diagnosis support, such as insulin/proinsulin autoantibodies (IAA/PAA), glutamic acid decarboxylase autoantibodies (GADA), and protein tyrosine phosphatase IA-2 autoantibodies (IA-2A) [8–10]. ZnT8A is detected in 60%–80% of juvenile-onset diabetic patients, and increases overall autoantibody positivity by 5% among those patients who were negative for the previously defined humoral markers, diminishing the number of cases of type 1B or idiopatic diabetes. ZnT8A could also be found in latent autoimmune diabetes of adults (LADA) contributing to the characterization of these patients [11].

ZnT8 is a 369 amino-acid protein coded by the gene *SLC30A8* in chromosome 8q24.11 [2]. Sladek et al. [12] demonstrated a strong association of a non-synonimus polymorphism at residue 325 (Arg/Trp) with type 2 diabetes. The fact that the major epitope for ZnT8A lies within the cytoplasmic domain (residues 268–369) and the variant residue at aa 325 is located at the most distal extension of the molecule into the cytoplasm as it was defined for young type 1 diabetic patients prompted us to determine whether there is any difference in the recognition of Arg or Trp in aa 325 within ZnT8 in adult-onset diabetic patients. The goal of this study was to evaluate the prevalence of ZnT8A in adult-onset patients initially classified as type 2 diabetic patients and to characterize the epitopes recognized by these autoantibodies. Finally, we aim to establish its potential use as an additional marker of autoimmunity in LADA patients.

Methods

General details

Blood samples were collected after overnight fasting and sera were stored at -20°C until assayed.

Nondiabetic control sera, used to establish a cut-off value, were from healthy subjects without personal or family history of autoimmune disease. The collection of serum samples was approved by the Ethics Committee of the Clinical Hospital José de San Martín.

Adult-onset diabetic patients

A population of 271 subjects attending the Division of Diabetes at the Clinical Hospital José de San Martín, Buenos Aires, Argentina, from 2002 to 2004, diagnosed for diabetes at age >30 years old (mean age 53.4 ± 10.9), body mass index (BMI) ≤ 30 (24.6 ± 2.9), without insulin treatment for the first year of disease, and initially classified as type 2 diabetic patients, were included in this study. Diagnosis was done according to the American Diabetes Association [13]. Relevant clinical information is given in Table I, and the criteria for oral hypoglycaemic agent therapy were those of the UK Prospective Diabetes Study [14]. Then, 101 patients were prospectively followed during 6 years from the date of diagnosis for the development of insulin requirement. The patients included in this study had not been treated with insulin before blood sample collection for immunochemical analysis. All subjects were informed of the purpose of the study, and their consent for study participation was obtained.

ZnT8A assays

ZnT8A was determined by radioligand binding assay (RBA) using C-terminal domains (residues 268–369) carrying 325Arg, 325Trp, and a dimeric cDNA construct carrying 325Arg–325Trp codified by cDNA plasmids, provided by Dr J. Hutton from the Barbara Davis Center of Childhood Diabetes, University of Colorado, Aurora, CO, USA. These constructs were used to analyze the epitope specificity.

ZnT8–Arg325, ZnT8–Trp325, and ZnT8–Arg–Trp325 were transcribed and translated using a rabbit reticulocyte lysate system (Promega, Madison, WI, USA) and ^{35}S-methionine (New England Nuclear, Boston, MA, USA) according to the manufacturer's instructions. Translation products were diluted in RBA buffer (0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4, 0.15% Tween 20, 0.1% Aprotinin, and 0.1% bovine serum albumin) and applied to a PD10 column (GE, Healthcare BioScience, Upsala, Sweden) to remove the free ^{35}S-methionine. Aliquots of 5 μl of human sera were incubated overnight at 4°C with 20,000 cpm of the different tracers (^{35}S-ZnT8–Arg325, ^{35}S-ZnT8–Trp325, or ^{35}S-ZnT8–Arg–Trp325) in a final volume of 60 μl in RBA buffer. Subsequently, 50 μl of 50% protein A-Sepharose 4B FF (GE, Healthcare BioScience) in RBA buffer was added, and the suspension was incubated for 2 h at room temperature on an end-over-end shaker. The samples were centrifuged and the supernatant was discarded. The pellets were washed three times with 200 μl of RBA buffer containing 0.35 M NaCl, suspended in 100 μl 1% Sodium dodecyl sulfate (SDS) and

Table I. Clinical and laboratory data for 271 adult-onset diabetic patients.

Patients (*n*)	271
Gender (F:M)	104:167
Mean age at diagnosis (year)	53.4 ± 10.9
Age range (year)	30–84
Mean BMI at diagnosis	24.6 ± 2.9
A1C (%)	8.0 ± 2.2
Fasting glucose (mg/dl)	200.6 ± 85.6
Triglycerides (mg/dl)	164.3 ± 115.1
HDL cholesterol (mg/dl)	48.6 ± 13.9
Total cholesterol (mg/dl)	207.2 ± 43.9

centrifuged for 5 min at 6000*g*. The supernatants were carefully transferred to vials for scintillation counting. Results were expressed as $B\% = 100 \times$ bound cpm/total cpm and expressed as SD score $= (B - B_C\%)/SD_C$, where $B_C\%$ is the control mean $B\%$ and SD_C its SD. Thirty normal controls were included in each assay and $B_C\%$ was normally distributed. An assay was considered positive if SD score > 3. In the second international workshop on ZnT8A held in 2009 by the Diabetes Autoantibody Standardization Program (DASP), the assay for ZnT8–Arg325, ZnT8–Trp325, and ZnT8–Arg–Trp325 showed 22%, 26%, and 60% sensitivity and 100%, 98.8%, and 96.5% specificity, respectively. The inter-assay coefficient of variation (CVs) in triplicates were 31.2%, 19.8%, and 29.3% for an SD score of 37.3, 23.5, and 49.1 for ZnT8–Arg325, ZnT8–Trp325, and ZnT8–Arg–Trp325, respectively.

Detection of other anti-islet autoantibodies

GADA and IA-2A were determined by RBA essentially as previously described [15]. In brief, cDNA coding for human GAD65 or tyrosine-phosphatase IA-2A was transcribed and translated using a rabbit reticulocyte lysate system in the presence of ^{35}S-methionine as indicated for the ZnT8A assay. The RBA was carried out by incubating 2.5 µl of human sera overnight at 4°C with 10,000 cpm of the ^{35}S-GAD or ^{35}S-IA-2A in a final volume of 60 µl in RBA buffer.

Subsequently, isolation of immunocomplexes and wash steps were done as described for ZnT8A assay. Results were expressed as SD score. In the DASP 2007, the GADA and IA-2A assays had sensitivities of 80% and 69% and specificities of 98% and 97%, respectively. PAA was also determined by RBA as described by Valdez et al. [16]. The cDNA coding for human PI was transcribed and translated using a rabbit reticulocyte lysate system in the presence of ^{35}S-cysteine, according to the manufacturer's instructions. After overnight refolding, favored by a disulfide reduction–reoxidation procedure, ^{35}S-PI was isolated by reverse-phase HPLC. Sera (30 µl) were incubated for 7 days at 4°C with 1000 cpm of ^{35}S-PI in 90 µl of RBA buffer. Subsequently, isolation of immunocomplexes and wash steps were done as indicated previously. Results are expressed as SD score.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism software, version 4.00. Results were expressed as mean ± SD unless otherwise indicated. Differences in non-parametric data were tested by the Mann–Whitney test. A *P*-value less than 0.05 was considered statistically significant.

Results

ZnT8A prevalence and presence of other islet autoantibodies

First, 271 adult-onset diabetic patients were tested in parallel for IAA/PAA, GADA, IA-2A, and ZnT8A (values were composited by reactivity to ZnT8–Arg325, ZnT8–Trp325, and ZnT8–Arg–Trp325). Results are shown in Figure 1. Out of these patients, 60 (22.1%) presented at least 1 humoral marker, 7 (2.6%) were PAA +, 34 (12.5%) were GADA +, 9 (3.3%) were IA-2A +, and 29 (10.7%) were ZnT8A + (Figure 1A).

Figure 1B shows the isolated and overlapping prevalence of PAA, GADA, IA-2A, and ZnT8A. Double positivity was 3 (1.1%) for PAA–GADA, 2 (0.7%) for GADA–IA-2A, 1 (0.4%) for PAA–ZnT8A, and 4 (1.4%) for GADA–ZnT8A. Triple positivity was 1 (0.4%) for PAA–GADA–ZnT8A and 3 (1.1%) for GADA–IA-2A–ZnT8A. Twenty-one (7.7%), 3 (1.1%), 2 (0.7%), and 19 (7.0%) patients were positive for GADA, IA-2A, PAA, and ZnT8A alone, respectively.

Autoantibody reactivity to ZnT8 variants

Among the 29 ZnT8A positive patients, 2 (7.0%) had autoantibodies against ZnT8–Arg325, 15 (51.7%) against ZnT8A–Trp325, and 18 (62.1%) presented autoantibodies against the dimeric construct ZnT8–Arg–Trp325 (Figure 2A).

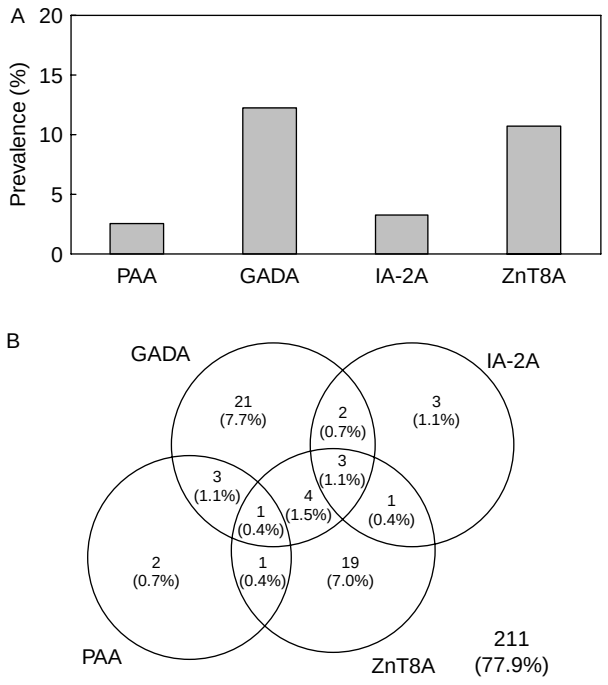

Figure 1. Autoimmune markers in adult-onset diabetic patients ($n = 271$). (A) Prevalence of autoimmune markers and (B) marker profile, represented in Venn diagrams.

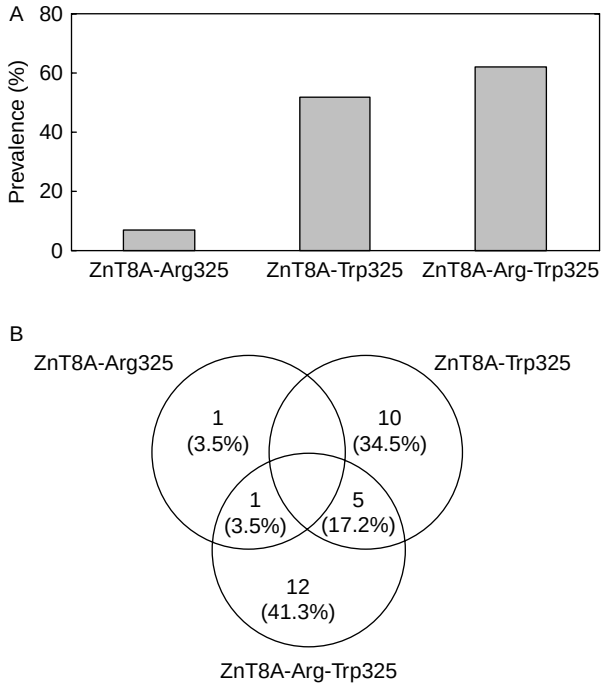

Figure 2. Autoantibody reactivity to ZnT8 aa325 variants. (A) Prevalence of ZnT8A–Arg325, ZnT8A–Trp325, and ZnT8A–Arg–Trp325 and (B) ZnT8A profile, represented in Venn diagrams, in adult-onset diabetic patients ZnT8A positive ($n = 29$).

Seventeen adult patients had antibodies only to 1 of the ZnT8-325 variants, that is to say that those samples which recognized ZnT8–Arg325 did not recognize ZnT8–Trp325 and vice versa. In addition, there were 12 patients who had antibodies against the chimeric construct ZnT8–Arg–Trp325, but were not reactive to any of the other monomeric constructs analyzed (ZnT8–Arg325 or ZnT8–Trp325). This suggests that the dimeric chimera must be exhibiting an epitope that is not present in either of the monomeric antigens (Figure 2B).

ZnT8As and other autoantibodies associated with clinical phenotype

Within the original group of marker positive patients, the prevalence of ZnT8A (48.3%) was in second place after GADA (56.7%; Table II). When comparing the clinical phenotype of patients with ZnT8A as the only humoral marker versus patients with GADA as the only humoral marker, no significant differences were found in age at diagnosis, Hemoglobin A1c, BMI, and fasting glucose. The clinical phenotype analyzed according to the presence of at least one marker showed a trend of association with younger age at diagnosis and more prominent features of insulin deficiency (higher fasting glucose and lower BMI; Table II).

Among the 101 patients who were prospectively followed during 6 years, 18 (17.8%) required insulin treatment and between them, 10 (55.6%) presented

Table II. Clinical characteristics of adult-onset diabetes according to the number of autoantibodies.

	No Abs	At least 1 Ab	Multiple Abs	*P* value no Ab vs. at least 1 Ab	*P* value no Ab vs. multiple Abs	GADA alone	ZnT8A* alone	*P* value GADA alone vs. ZnT8A* alone
N	211	60	15			21	19	
Gender (F:M)	83:128	23:37	8:7			8:13	4:15	
Age at diagnosis	54.4 ± 10.9	49.9 ± 10.2	44.7 ± 5.4	<0.05	<0.05	47.2 ± 8.6	55.3 ± 10.2	<0.05
Hemoglobin A1c (%)	7.9 ± 2.2	9.0 ± 2.2	9.6 ± 2.4	NS	NS	8.2 ± 2.8	9.7 ± 1.3	NS
BMI	24.9 ± 3.0	23.8 ± 2.8	22.4 ± 2.2	<0.05	<0.05	24.7 ± 3.5	24.5 ± 2.4	NS
Triglycerides (mg/dl)	154.5 ± 108.2	207.8 ± 133.8	130.3 ± 18.6	NS	NS	193.9 ± 122.1	225.2 ± 185.7	NS
HDL cholesterol (mg/dl)	49.2 ± 14.6	46.0 ± 9.3	55.7 ± 7.7	NS	NS	44.1 ± 6.4	42.8 ± 10.6	NS
Total cholesterol (mg/dl)	206.3 ± 42.8	211.2 ± 48.0	199.7 ± 29.5	NS	NS	213.7 ± 59.9	204.3 ± 46.4	NS
PAA		7 (11.7%)	5 (33.3%)					
GADA		34 (56.7%)	13 (86.7%)					
IA-2A		9 (15.0%)	6 (40.0%)					
ZnT8A*		29 (48.3%)	10 (66.7%)					

Note: Data are expressed as mean ± SD or number (percentage); * values are composited by reactivity to ZnT8–Arg325, ZnT8–Trp325, and ZnT8–Arg–Trp325.

humoral autoreactivity. Over 83 patients without insulin requirement, 19 (22.9%) had at least 1 autoantibody. Figure 3 shows the 29 patients who had autoimmunity showing the overlapping prevalence of humoral markers and the association with insulin treatment.

Discussion and conclusion

Until recently, most screening tests for adult-onset autoimmune diabetes were based mainly on the detection of GADA complemented with other markers assessment. Our study shows that ZnT8A is a humoral marker that must be included when screening LADA patients. It is accepted that adult diabetes-associated antibodies largely recognized the C-terminal of the antigen (residues 268–369), whereas antibodies against N-terminal moiety (residues 1–74) were rare. Moreover, Wenzlau et al. [17] described that approximately 80% of ZnT8A positive sera from type 1 diabetic patients exclusively recognized epitopes contained within the final 102 amino acids of the protein. Accordingly, in this study, we determined ZnT8A for specific epitope recognition in sera from presumptive type 2 diabetic patients by using C-terminal ZnT8 constructs carrying 325-Trp or 325-Arg and a dimeric construct carrying both 325-Trp and 325-Arg.

In a previous report [11], it was demonstrated that 1.4% of patients with adult-onset diabetes initially identified as "marker negative" based on GADA and IA-2A screening when tested for ZnT8A were reclassified as autoantibody positive. In this study, we confirmed such a profile, although exclusive ZnT8A positivity was more frequent (7.0%). However, it is important to take into account that the inclusion criteria used in our study were different, since adult-onset diabetic patients were recruited with BMI ≤ 30. Besides, ZnT8A was investigated employing the three constructs (ZnT8–Arg325, ZnT8–Trp325, and ZnT8–Arg–Trp325). The preceding reasons could explain the relative high percentage of patients only positive for ZnT8A in the present report.

It is important to identify better predictive markers associated with disease progression in order to achieve an early metabolic control and thus, avoiding diabetic complications. To further extend our analysis, we compared the clinical phenotype of the groups of patients presenting GADA as the single humoral marker versus those having only ZnT8A, not finding significant differences between groups, thus indicating the homogeneity of their clinical characteristics.

However, when compared with antibody-negative individuals, patients with at least one humoral marker exhibit younger age at disease onset, higher blood glucose, and lower BMI ($P < 0.05$; Table II). This could be associated with early insulin requirement as was suggested by Kawasaki et al. [18]. On the other hand, there was no relationship between ZnT8A levels and BMI, despite the isoform used in the assays. This was analyzed by lineal regression in plots of BMI versus ZnT8A levels in which slopes had no significant deviation from zero.

When prospectively following a subgroup of patients during 6 years, we observed that 17.8% required insulin treatment, 55.6% of whom had at least one autoantibody. Further, studies must be done to correlate the autoimmune humoral profile with future β-cell failure and hence, insulin requirement.

In this study, we demonstrated that a significant proportion of autoimmune adult-onset patients, initially classified as type 2 diabetic patients, presented ZnT8A as the only humoral marker. Moreover, between them, the higher prevalence was observed for ZnT8A–Trp325 (51.7%; Figure 2). Although the reason for this is unknown, it presumably reflects interindividual variation in the diabetogenic process.

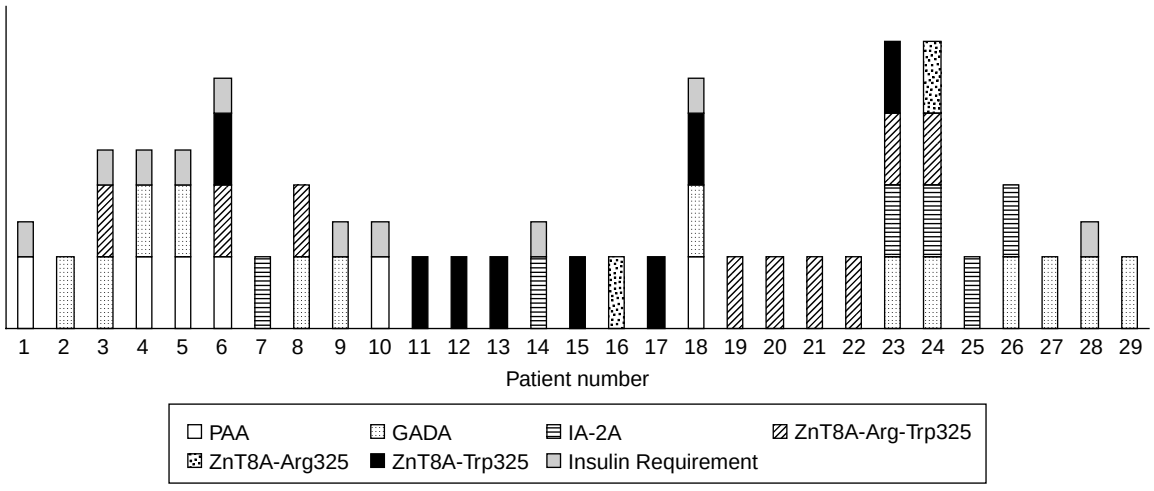

Figure 3. Marker profile and association with insulin requirement in 29 patients with humoral immune response against β cell antigens.

Further analysis should be carried out in order to establish whether there is any correlation between the frequency of ZnT8A against the Trp325 isoform and the polymorphism in the population studied.

Our present results strongly support the idea that screening for LADA patients is best carried out if a more complete spectrum of humoral response anti-β cell and its products is routinely assessed. In this sense, a minimal set of marker determination must include at least the screening of GADA plus ZnT8A–Arg–Trp325. In conclusion, we found a relative high frequency of ZnT8A marker positive within the Argentinian diabetic patients, initially classified as type 2, which in fact must be diagnosed as LADA, indicating the importance to include such marker assessment to the conventional panel of test.

Acknowledgments

We are grateful to J. Hutton from the Barbara Davis Center of Childhood Diabetes, University of Colorado, Aurora, CO, USA, for the gift of cDNA plasmids encoding the COOH-terminal fragment (aa 268–369) of the ZnT8 aa 325-Arg allele, the ZnT8 aa 325-Trp allele, and a chimeric construct encoding both alleles in combination.

Declaration of interest: This work was supported in part by grants from FONCYT Programme of the Agency for Science and Technology Promotion (ANPCyT), National Research Council (CONICET), and the University of Buenos Aires, Buenos Aires, Argentina. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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