

Zinc Transporter 8 antibodies complement GAD and IA-2 antibodies in the identification and characterization of adult-onset autoimmune diabetes.
NIRAD 4

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Objective: Zinc Transporter 8 (ZnT8) is an islet beta-cell secretory granule membrane protein recently identified as an autoantibody antigen in type 1 diabetes. The aim of this study was to determine prevalence and role of antibodies to ZnT8 (ZnT8A) in adult-onset diabetes.

Research design and methods: ZnT8A were measured by a radio immunoprecipitation assay using recombinant ZnT8 COOH-terminal or NH₂-terminal proteins in 193 patients with adult onset autoimmune diabetes as having antibodies to either GAD (GADA) or IA-2 (IA-2A) and in 1056 antibody-negative patients with type 2 diabetes from the NIRAD study.

Results: ZnT8A-COOH were detected in 18.6% patients with autoimmune diabetes and 1.4% with type 2 diabetes. ZnT8A-NH₂ were rare. ZnT8A were associated with younger age and high GADA titre. The use of GADA, IA-2A and ZnT8A in combination allowed a stratification of clinical phenotype, with younger age of onset and characteristics of more severe insulin deficiency (higher fasting glucose and HbA1c, lower Body Mass Index, total cholesterol, triglycerides) in patients with all three markers, with progressive attenuation in patients with two, one and no antibodies (all P for trend <0.001). Autoantibody titres, association with high risk HLA genotypes and prevalence of thyroid peroxidase antibodies followed the same trend (all P<0.001).

Conclusions: ZnT8A are detectable in a proportion of adult-onset autoimmune diabetes and appear as a valuable marker to differentiate clinical phenotypes.

Zinc Transporter 8 (ZnT8) is a pancreatic beta-cell secretory granule membrane protein that has been recently identified as a target of humoral immunity in type 1 diabetes (1). Autoantibodies to ZnT8 (ZnT8A) constitute an additional marker of autoimmune diabetes, which complement the established antibodies to insulin (IAA) (2), glutamic acid decarboxylase (GADA) (3), and protein tyrosine IA-2 (IA-2A) (4). In their first report, ZnT8A were detected in 63% of young patients at onset of disease, overlapping with, but also independent of GADA, IAA and IA-2A, and the combined use of these four antibody markers raised the detection rate of autoimmunity up to 94% in new onset cases. Moreover, ZnT8A could be detected also in the preclinical phase of type 1 diabetes, showing a trend to a later appearance relative to IAA, GADA and IA-2A, but with the ability to identify individuals with a more rapid progression to clinical disease.

Although islet autoimmunity is responsible for the large majority of childhood and adolescent-onset diabetes, it can be found also in 4 to 10% of adult-onset diabetes. This sub-group of patients tests positive for humoral markers of islet autoreactivity, despite having clinical features indistinguishable from those of classical type 2 diabetes and is referred to as LADA (Latent Autoimmune Diabetes of Adult). LADA patients are solely identified by the detection of circulating islet autoantibodies, with Islet Cell Antibodies (ICA) and GADA being the antibody markers with the highest prevalence (5)(6), followed by IA-2A antibodies, that are detected in a minority of cases and almost invariably associated with GADA (7), while insulin autoantibodies, that constitute a specific marker of juvenile diabetes inversely related to age and rare in adults, are unlikely to be useful for LADA screening (8)(9)(10). The aim of this study was to evaluate the

prevalence of ZnT8A in adult onset diabetes and establish their potential use as additional marker of autoimmunity and phenotype characterization in this patient population.

RESEARCH DESIGN AND METHODS

Patients. All patients investigated participated to the Non Insulin Requiring Autoimmune Diabetes (NIRAD) study, a nationwide survey based in Italy, conducted with the aim of assessing the prevalence and characteristics of adult-onset autoimmune diabetes (11). Inclusion criteria were: a) diagnosis of Diabetes mellitus according to the American Diabetes Association, with no insulin requirement and no evidence of ketosis from diagnosis to screening time; b) disease duration between 6 months and 5 years. Exclusion criteria included prior insulin therapy, pregnancy and the presence of any other severe disease. The study was approved by the Ethics Committees of all participating Centers and a written informed consent was obtained by all patients prior to screening. Of the original NIRAD cohort of 4250 subjects with adult-onset initially non insulin-requiring diabetes we studied all 193 patients (4.5% overall prevalence) with autoimmune diabetes defined as having antibodies to either GAD (GADA) or IA-2 (IA-2A) (age 50.3 ± 12.8 yr; mean duration of diabetes 2.3 years, range 0.6 – 4.8 years) (11) and 1056 (age 51.8 ± 11.8 yr, mean duration of diabetes 2.4 years, range 0.5 – 5 years) patients with type 2 diabetes. For the comparison of clinical phenotypes a subset of 348 age and sex matched antibody-negative patients with type 2 diabetes (age 51.1 ± 10.8 yr; mean duration of diabetes 2.2 years, range 0.5 – 5 years) was selected. Previous patient assessment included the following measurements: anthropometrics; fasting glucose, total cholesterol, HDL cholesterol, triglycerides, uric acid and glycated haemoglobin; GADA, IA-2A and thyroid peroxidase (TPO)

antibodies; HLA-DRB1 and DQB1 typing (11)(12). The distribution of GADA titres in patients with autoimmune diabetes was independent of diabetes duration and showed a bimodal distribution. Consistently with this observation, patients with autoimmune diabetes were divided into subgroups representing the two distributions, namely low (≤ 32 arbitrary units, equivalent to 300 WHO units) and high (> 32 arbitrary units) GADA titres (11)(13).

ZnT8 cDNA cloning. Total RNA was extracted from isolated human pancreatic islets with the Mirvana™ kit (Applied Biosystems, Foster City, CA, USA) according to the total RNA isolation protocol and reverse transcribed to cDNA using the Superscript III™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). The coding regions of ZnT8 corresponding to aminoacids 1-74 (ZnT8-NH2) and amino-acids 268-369 (ZnT8-COOH R325) were amplified by PCR using PfuUltra™ II Fusion HS DNA Polymerase (Stratagene, La Jolla, CA, USA) with specific primers containing Eco RI restriction sites and for the forward primers an in frame start codon within the context of a canonical Kozak sequence. Amplified PCR products were purified with Montage-PCR filter units (Millipore, Billerica, MA, USA) and cut with the restriction enzyme Eco RI (Roche Diagnostics, Basel, Switzerland). Digested PCR fragments were purified with MicropureEZ spin columns (Millipore), ligated into the EcoRI site of the pTnT™ plasmid vector (Promega, Hercules, CA, USA) and transformed by electroporation in TOP10 E. Coli bacterial cells. Plasmid DNA was extracted from the obtained clones with Genelute™ spin columns (Sigma-Aldrich, St Louis, MO, USA) and the cDNA insert verified by sequencing on an ABI3130 automated sequencer (Applied Biosystems). For large scale plasmid DNA preparations Qiagen midi columns were used (Qiagen,

Hilden, Germany). A clone containing a cDNA encoding for the polymorphic residue tryptophane in position 325 of ZnT8 (ZnT8-COOH W325) was obtained from the ZnT8-COOH R325 by site directed mutagenesis according to the QuickChange™ protocol (Stratagene).

ZnT8A antibody assay. Antibodies to ZnT8 (ZnT8A) in patient sera were measured by immunoprecipitation of radiolabelled recombinant ZnT8 antigens. ZnT8 ZnT8-NH2 and ZnT8-COOH proteins were expressed *in vitro* in a rabbit reticulocyte lysate using the TNT® Quick Coupled Transcription/Translation System SP6 kit (Promega) in the presence of 40 μ Ci of 35 S labelled methionine (Perkin Elmer, Waltham, MA, USA), purified by size exclusion chromatography on NAP-5™ columns (GE healthcare Bio-Sciences, Uppsala, Sweden) and the recovered radioactivity measured on a TopCount™ beta counter (Perkin Elmer). For immunoprecipitation 20.000 cpm of recombinant radiolabelled ZnT8-NH2, when testing for ZnT8A-NH2 autoantibodies, or a mixture of 10.000 cpm each of ZnT8-COOH R325 and W325 antigens, when testing for ZnT8A-COOH autoantibodies, were added in 25 μ l of tris-buffered saline pH 7.4, 0.1% tween 20 (TBST) to 2 μ l of human serum for each test sample in duplicate wells of a polystyrene 96 deep well plate (Beckman Coulter, Fullerton, CA, USA) and incubated overnight at 4 °C. Immune complexes were recovered by the addition of 4 μ l resuspended CL4B protein-A sepharose (GE healthcare Bio-Sciences) in 50 μ l of TBST and incubated with agitation at 4 °C for 1 hour. Protein-A sepharose beads were then washed 5 times by adding 750 μ l of TBST followed by centrifugation at 700g for 3 min and buffer removal by aspiration. After the last wash protein-A sepharose beads were resuspended and transferred to the wells of a 96 well optiplate (Perkin Elmer), added with 150 μ l of Microscint 40™ scintillation fluid (Perkin

Elmer) and the recovered radioactivity measured by counting each well for 5 min on TopCount™ beta counter. Results were expressed in arbitrary units derived by a standard curve made of serial dilutions of a positive serum included in each assay run. Threshold for positivity was placed at the 99th percentile of a 100 non diabetic controls. The assay for ZnT8A-COOH showed an inter-assay CV of 8.4% and an intra assay CV of 5.5% while the assay for ZnT8A-NH₂ showed an inter-assay CV of 9.5 % and an intra assay CV of 6.3%. In the second international workshop on ZnT8A held in 2009 by the Diabetes Autoantibody Standardization Program the assay for ZnT8A-COOH antibodies showed a lab reported sensitivity of 68%, a specificity of 99%, an area under the ROC curve of 0.8848 and an adjusted sensitivity at 95% specificity of 74%. The assay for ZnT8A-NH₂ in the first international workshop on ZnT8A held in 2007 showed an 11% sensitivity and 99% specificity.

Statistical analysis. Statistical analysis was performed using SPSS statistical software, version 13 (SPSS, Illinois, USA). Data are expressed as frequencies, as means ± standard deviation (SD) or as median ± interquartile range. Frequency differences were compared using the χ^2 test (with Yates' continuity correction) or Fisher's exact test when appropriate. The exploration of statistical differences between groups for quantitative variables was investigated using multiple linear regression. Comparisons were adjusted for age of recruitment, duration of disease, gender and therapy. The non parametric Mann-Whitney test was used to investigate the relation between TPO titers (Units) and number of antibodies. Data for triglycerides, HDL and ZnT8A titers (Units) were transformed using log base 10 to normalize their distributions. HLA DQB1 and DRB1 allele frequencies were in Hardy-Weinberg equilibrium (i.e., observed and

expected genotype frequencies did not differ significantly). HLA class II alleles were evaluated as previously described (11).

RESULTS

Prevalence of ZnT8A. As previously reported, of the 193 patients with autoimmune diabetes identified within the NIRAD Study (4.5% prevalence of adult onset diabetes), 191 had GADA and 39 had IA-2A; of these, 154 had GADA only, 2 had IA-2A only and 37 had both (9). ZnT8A-COOH were detected in 36/193 (18.6%) autoimmune (all 36 with GADA, 20 with both GADA and IA-2A) and 16/1056 (1.4%) patients with type 2 diabetes (fig.1); ZnT8A-NH₂ were rare and found only in 4/193 (2.1%) patients with autoimmune diabetes (3 having also ZnT8A-COOH) and 1/348 (0.3%) patient of the subset of matched patients with type 2 diabetes. Therefore, ZnT8A hereafter are intended as ZnT8A-COOH if not otherwise specified. The prevalence of ZnT8A within autoimmune patients was higher in younger patients and declined with age: in subjects 0-49 yrs: n=22 (12.2%) in subjects 49.1-58.8 yrs: n=12 (6.6%) and in subjects > 58.9 yrs n=8 (4.4%) (p for trend 0.0058).

Within the originally defined autoimmune diabetes group, 20 patients resulted positive for 3 autoantibodies, 33 for 2 antibodies (17 with GADA and IA-2A, 16 with GADA and ZnT8A) and 140 for 1 antibody only (138 with GADA, 2 with IA-2A) (fig. 2).).

ZnT8A, other autoantibodies and clinical phenotype. Within the original group of autoimmune patients (with either GADA and/or IA-2A), those having ZnT8A had more frequently associated high GADA titers (greater than 32 arbitrary units, equivalent to 300 WHO units) (11) and IA-2A (both P<0.01 vs ZnT8A-negative).

However, within the high GADA titers group no statistically significant differences in clinical features were observed between ZnT8A positive and negative patients.

The clinical phenotype analyzed according to the number of islet autoantibodies showed a trend towards younger age at diagnosis, more prominent features of insulin deficiency (higher fasting glucose and HbA1c; lower BMI, waist circumference, total cholesterol, triglycerides and uric acid) and higher prevalence of associated TPO antibodies proportional to the number of autoantibodies (all P for trend <0.001). All these distinguished traits, although attenuated and with the exception of total cholesterol and triglycerides, remained significantly different in patients with a single autoantibody compared to those with classical type 2 diabetes (all P ≤0.04). Accordingly, the titers of GADA, IA-2A and ZnT8A and the prevalence and titers of associated TPO antibodies, as well as the association with high risk HLA genotypes followed the same trend (all P<0.001) (table 1).

CONCLUSIONS

This study shows that ZnT8A, recently identified as autoantibodies associated with juvenile onset type 1 diabetes, are also a marker of adult onset autoimmune diabetes.

Adult diabetes associated antibodies largely recognized the COOH terminal of the antigen (amino-acids 268-369), while antibodies against the NH₂-terminal moiety (aminoacids 1-74) were rare. Therefore, in adult diabetes ZnT8A essentially correspond to ZnT8-COOH antibodies. In the cohort of the NIRAD study, ZnT8A were detected in 18.6% of patients previously identified by GADA and/or IA-2A; the overall prevalence was similar to that of IA-2A, higher in younger patients and declining with age. In the original report, it was shown that the prevalence of ZnT8A was low in younger individuals, but increased dramatically from 3 year onwards, peaked at 80% in late adolescence and tended to decline thereafter (1). The present findings are consistent with the evidence of a further decrease of ZnT8A

prevalence by increasing age, being observed in ~12% of adult patients below 50 years of age and becoming very rare after the age of 60. In patients with adult onset diabetes previously identified as non-autoimmune based on GADA and IA-2A screening, testing for ZnT8A identified an additional 1.4% of subjects as autoantibody positive. This represents a marginal increase over the expected 1% of positives based on the threshold adopted in our assay and, if extrapolated to the whole of the NIRAD cohort, would bring the potential prevalence of autoantibody positive subjects in adult onset diabetes up from the original 4.5% (11) to 5.9%. It is debatable, however, whether positivity for a single low titre islet autoantibody other than GADA is a reliable indicator of autoimmune disease.

To further extend our analysis we correlated the clinical phenotype to the number of islet autoantibodies, a characteristic that has been demonstrated to reflect the intensity of autoimmune response and predict future insulin insufficiency (5)(7)(10)(14). The availability of ZnT8A as a marker additional to GADA and IA-2A allowed stratification across the intensity of islet autoimmune response which is clearly reflected by the clinical phenotype of patients with adult diabetes, with features of more severe insulin insufficiency proportional to the number and, accordingly, titers of islet autoantibodies. Indeed, when compared to antibody-negative type 2 diabetes, patients with multiple antibodies exhibited characteristics more similar to those of type 1 diabetes, with younger age at disease onset, higher blood glucose and HbA1c, lower BMI, waist circumference, cholesterol, triglycerides and uric acid and higher prevalence of associated TPO antibodies; most of these distinctive traits, although attenuated, were still present in patients with a single autoantibody, indicating that even a single marker of autoimmunity is able to distinguish

a degree of insulin insufficiency more severe than that of classical type 2 diabetes.

Unlike GAD and IA2, ZnT8 is highly β -cell-specific; therefore the presence of ZnT8A in patients from the NIRAD study demonstrates that antigens exclusively expressed in pancreatic beta cells are targets of the autoimmune process also in adult onset diabetes and support the usefulness of ZnT8A measurement in already identified single GADA positive adult patients to define patients with a more severe and islet specific autoimmunity.

In conclusion, ZnT8A are detected in a significant proportion of adult-onset autoimmune diabetes and appear as a valuable

marker to differentiate clinical phenotypes within this patient population.

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Figure Legends:

Figure 1: ZnT8A directed against the COOH domain are more frequent in high titer GADA positive patients. Filled symbols indicate positivity also for IA-2A. Dotted line indicates threshold for positivity.

Figure 2: Venn diagram of autoantibody combinations in autoantibody positive patients with adult onset diabetes

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Figure 1

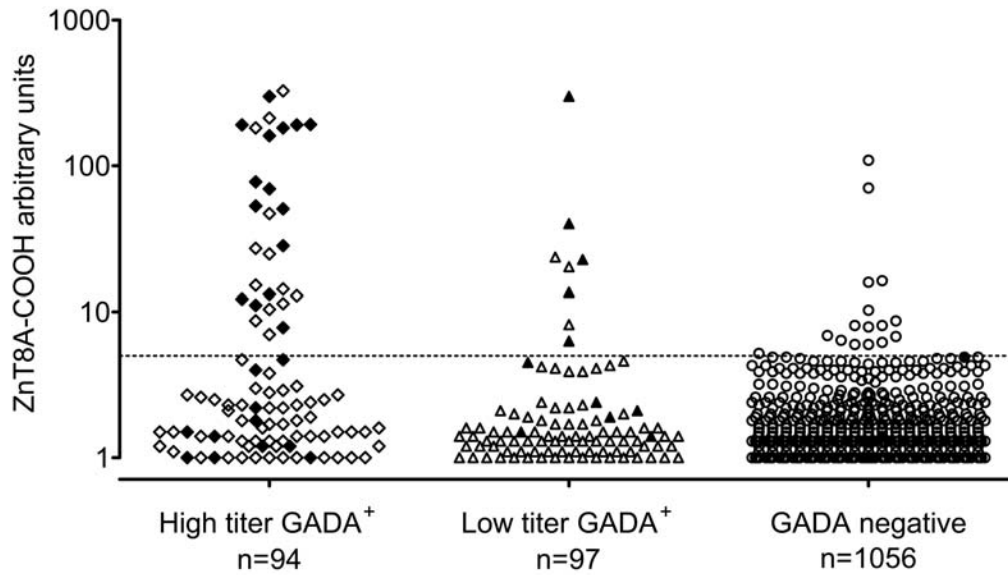


Figure 2

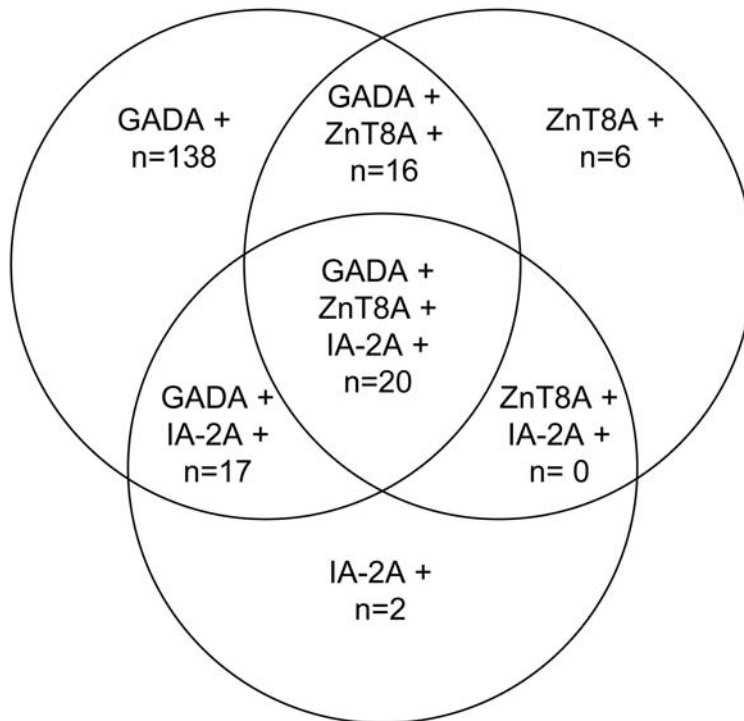


TABLE 1. Clinical characteristics of adult onset diabetes according to the number of autoantibodies.

	3 Ab	2 Ab	1 Ab only	None (Type 2)	p value for trend	p 1 Ab vs none	p excluding type 2
N=	20	33	140	342			
Sex (Males/Females)	10/10	20/13	71/69	174/168			
Age of diagnosis (years)	43.6 ± 16.4	45 ± 12.6	52.4 ± 11.2	51.6 ± 10.6	<0.0001	0.04	0.003
A1C (%)	8.2 ± 2.7	8.1 ± 2.2	7 ± 1.9	6.5 ± 1.4	<0.0001	0.01	0.03
BMI (kg/m2)	24.2 ± 4.5	25.5 ± 4.4	27.6 ± 4.8	29.4 ± 5.1	<0.0001	0.004	0.002
Waist circumference (cm)	84.8 ± 8.17	91.3 ± 13.3	96.2 ± 13.2	98 ± 13.1	<0.0001	0.03	0.003
Fasting glucose (mg/dl)	184 ± 65	163 ± 41	166 ± 55	144 ± 48.9	<0.0001	0.001	ns
Triglycerides (mg/dl)	83 (54-148)	110 (76-182)	128 (83-199)	135 (101-196)	0.0008	ns	0.03
HDL cholesterol (mg/dl)	46.5 (39-56)	50.5 (44-59)	48 (40-56)	45 (38-56)	ns	ns	ns
Total cholesterol (mg/dl)	168 ± 49	195 ± 43	205 ± 44	206 ± 43	0.006	ns	0.04
Uric acid (mg/dl)	3.8 ± 1.2	4.7 ± 1.79	4.8 ± 1.4	5.16 ± 1.4	0.003	0.01	0.04
TPO-Ab ⁺ number (%)	9 (45%)	6 (18.2%)	37 (26.4%)	36 (10.5%)	<0.0001	<0.0001	ns
DRB1*03-DQB1*0201	7 (35%)	15 (45.4%)	52 (37.1%)	58 (17%)	<0.0001	<0.0001	ns
DRB1*04-DQB1*0302	5 (25%)	8 (24.2%)	34 (24.3%)	35 (10.2%)	0.0001	0.0001	ns
High-risk HLA genotypes*	2 (10%)	2 (6%)	12 (8.6%)	4 (1.1%)	<0.0001	0.0002	ns
Moderate-risk HLA genotypes [†]	5 (25%)	8 (24.2%)	33 (23.6%)	38 (11.1%)	<0.0001	0.0009	0.03
Low-risk HLA genotypes [‡]	13 (65%)	21 (63.6%)	95 (67.8%)	300 (87.7%)	<0.0001	0.0001	ns
ZnT8A titer	99 (13-191)	33 (6-110)	1.2 (1-1.5)	1.82 (1-2.28)	<0.01	ns	<0.0001
GADA titer	89 (20-130)	90 (25-98)	14 (8-90)		<0.01 [§]		
IA-2A titer	0.12 (0.04-0.25)	0.18 (0.01-0.31)	-0.04 (-0.08- 0.16)	-0.001 (-0.01- 0.08)	p <0.01	ns	
TPO-Ab+ titer	1781 (244-3120)	2850 (780-3250)	2000 (600-4650)	84 (65-432)	p <0.01 [¶]	ns	ns

*High: DRB1*03-DQB1*0201/DRB1*04-DQB1*0302 genotype (DRB1*04 different from 0403, 06, 11).

[†]Moderate: DRB1*04-DQB1*0302/DRB1*04-DQB1*0302, DRB1*03-DQB1*0201/DRB1*03-DQB1*0201, DRB1*04-DQB1*0302/X, and DRB1*03/X (X different from DRB1*03, DRB1*04-DQB1*0302 [DRB1*04 not 0403, 06, 11], or DQB1*0602/03) genotypes.

[‡]Low: other genotypes.

[§] p value for 1 vs 2 or vs 3 antibodies

^{||} p value for 3 or 2 vs 1 or vs None antibodies

[¶] p value for trend 3 or 2 or 1 vs None antibodies

Data are means ± SD except for antibody titres where median ± interquartile range are reported. All p values are for trend