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ZnT8 autoantibody prevalence is low in youth with type 2 diabetes and associated with higher insulin sensitivity, lower insulin secretion, and lower disposition index

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ABSTRACT

Aim: ZnT8 autoantibody positivity (ZnT8+) is associated with risk for type 1 diabetes and with metabolic complications in adults. Our aim was to assess prevalence of ZnT8 + in the Treatment of T2D in Adolescents and Youth (TODAY) cohort and describe associated phenotypic outcomes.

Methods: TODAY participants were 13.98 ± 2.03 years with a confirmed diagnosis of T2D, BMI percentile of 97.69 ± 3.32 (64% female), and GAD- and IA2- at baseline. ZnT8 autoantibodies were measured at baseline and end of study.

Results: 3 of 687 participants (0.29%) were ZnT8 + and there was one conversion (0.15%) from ZnT8- to ZnT8 + during the study. ZnT8A + individuals had higher HbA1c, HDL and LDL cholesterol, and IL-1 β concentrations, and lower BMI, IL-6, and triglyceride concentrations compared to the TODAY cohort and ZnT8A- individuals. They also had higher insulin sensitivity with lower insulin secretion and disposition index, metabolically resembling T1D. All ZnT8 + participants experienced loss of glycemic control on randomized treatment, but exhibited lower rates of diabetic complications than other groups.

Conclusion: Given the low rate of complications in ZnT8 + individuals, ZnT8 likely does not impact the clinical course of the disease in this population.

Introduction

ZnT8 is a Y-shaped transmembrane protein that acts as a zinc pump to increase intracellular zinc concentrations and as a zinc sensor (see [1] for recent review). It is primarily located in pancreatic β -cells but can be detected at lower levels in other tissues, including pancreatic α -cells and cardiomyocytes. ZnT8 autoantibody positivity (ZnT8+) was present in 60–80% of individuals with new-onset type 1 diabetes (T1D) compared to < 2% of controls and < 3% of individuals with phenotypic type 2 diabetes (T2D) [2]. In addition, presence of ZnT8 + is associated with

increased risk of T1D and adding ZnT8 to the traditional T1D markers, glutamic acid decarboxylase 65 autoantibody (GAD), insulinoma-associated protein 2 autoantibody (IA2A), and insulin autoantibody (IA), increases autoimmunity detection rates to 98% [2].

In adults with T1D, ZnT8 + is associated with lower C-peptide concentrations and higher incidence of multiple diabetes-related antibodies [3]. In that study, participants who were under 35 years of age at diagnosis were more likely to exhibit multiple antibody positivity, which was rare in those diagnosed over 35 years. Similarly, in children with newly-diagnosed T1D, ZnT8 + is associated with older age at diagnosis,

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higher presence of multiple antibody positivity, and higher presentation at diagnosis with diabetic ketoacidosis [4]. ZnT8 + is related to more severe β -cell dysfunction in T1D and in adults with phenotypic T2D [5] and is associated with a 3.9-fold increased incidence of severe hypoglycemia [6]. Therefore, ZnT8 + is associated with older age, inflammation, and acute metabolic complications in adults with both T1D and phenotypic T2D.

There is substantial evidence that zinc transporters may play a role in the pathogenesis of diabetes. Although not specific to ZnT8, adult patients with T2D exhibit lower Zn transporter expression relative to controls and, unlike controls, plasma zinc concentration is unrelated to zinc transporter expression in those with T2D [7]. This led to the speculation that Zn dyshomeostasis is important in the pathogenesis of T2DM. In post-menopausal women with phenotypic T2D, those with ZnT8 + had higher HbA1c levels than those who were ZnT8- [8]. Both GAD and ZnT8 autoantibodies are associated with increased neuropathy and nephropathy in adults with phenotypic T2D, as well as increased adiposity [9], an independent risk factor for development of T2D.

Overall, these data indicate a role for ZnT8 in the pathogenesis and metabolic consequences of diabetes in adults. However, there are currently no data describing the prevalence or phenotypic effects of ZnT8 + in youth with phenotypic T2D who are otherwise pancreatic autoantibody negative. Therefore, the purpose of this analysis was to assess the baseline prevalence of ZnT8 + and longitudinal autoantibody conversion rates in the Treatment of Type 2 Diabetes in Adolescents and Youth (TODAY) cohort and describe associated diabetes and phenotypic outcomes.

Methods

Participants and study design

The TODAY study rationale, design, and methods have been described previously [10]. Briefly, eligibility criteria included age 10–17 years, diagnosis of diabetes consistent with type 2 diabetes with duration < 2 years, BMI \geq 85th percentile, fasting C-peptide levels > 0.6 ng/ml, and negative GAD and insulinoma-associated protein 2 (IA-2) antibodies. Exclusion criteria included diabetic ketoacidosis at any time after diagnosis, except for a single episode related to a significant intercurrent medical illness. All participants provided informed consent and minor children confirmed assent according to local guidelines before participation. The TODAY Study Group is composed of 15 clinical centers, a coordinating center, the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) project office, and central cores and laboratories (a list of the TODAY study centers and contributing investigators at each center and of industry support of the TODAY trial is found in an online appendix, available at <http://care.diabetesjournals.org/cgi/content/full/dc10-0373/DC1>). The protocol was approved by an External Evaluation Committee convened by NIDDK and by the institutional review board of each participating center. A Data and Safety Monitoring Board convened by NIDDK reviewed progress and safety regularly throughout the study.

Screening visits (n = 1211) were conducted from May 2004 to September 2008. Eligible participants were screened while using their current diabetes treatment, and rapid-acting insulin was held until after the assessment. The assessment included measurement of height, weight, and blood pressure. Race/ethnicity was determined by self-report using the 2000 Census collection format. A family history of diabetes and additional demographic data were obtained.

Laboratory studies were performed in the fasted state and included a lipid profile (total, LDL, and HDL cholesterol and triglycerides), C-peptide, IA-2 and GAD-65 autoantibodies, and HbA1C. All testing was performed by the Central Laboratory at the University of Washington.

Only patients with fasting C-peptide levels > 0.6 ng/ml and negative IA-2 and GAD-65 autoantibodies were eligible for participation in the TODAY study [10]. 592 participants had GAD and IA2 antibody

measurements available at post-screening time points and were included in this analysis. Antibodies were measured for each TODAY participant at screening and end of study. Participants who reached the primary outcome of loss of glycemic control before the end of study also had autoantibody testing repeated at time of failure. ZnT8 antibody testing became available after the screening period for TODAY ended, so stored samples were retrieved for ZnT8 testing. Not all participants had blood available for this testing, so the number of participants in the ZnT8 analysis was 687 from a total of 699 enrolled TODAY participants. We classified a participant as ZnT8 positive if the value was > 0.03 at baseline or at any point during participation in TODAY, on either a single occasion or on multiple occasions. Since the presence of IA2 or GAD autoantibody positivity was exclusionary for enrollment, we classified participants as IA2 or GAD autoantibody positive if they had at least one post-baseline positive value, whether a single occasion and on multiple occasions.

Laboratory methods

Samples were processed following standardized procedures and shipped either fresh or on dry ice as applicable to the Northwest Lipid Metabolism and Diabetes Research Laboratories, University of Washington (Seattle, WA). C-peptide was measured by a two-site immunoenzymatic assay on a dedicated instrument (Tosoh Bioscience, San Francisco, CA). The assay sensitivity is 0.05 ng/ml. HbA1C levels were determined by an automated nonporous ion-exchange high-performance liquid chromatography system (G7; Tosoh Bioscience).

Measurements of total cholesterol, triglycerides, and HDL cholesterol were performed enzymatically using a Roche reagent on a Roche Pmodule autoanalyzer. LDL cholesterol levels were calculated by the Friedewald equation for samples with triglycerides < 400 mg/dl and by the Lipid Research Clinic Beta Quantification approach for those with triglycerides \geq 400 mg/dl. Analysis of Interleukin-6 (IL-6) was performed using human high sensitivity magnetic beads-based method (EMD Millipore Inc., Billerica MA). The assay sensitivity was 0.18 pg/mL. The assay sensitivity was 0.9 pg/mL. The intra- and inter-assay CVs were 4% and 8.3%, respectively.

Concentrations of creatinine in serum and urine were determined using the Creatinine Plus enzymatic Roche reagent on a Modular P analyzer (Roche Diagnostics, Inc., Indianapolis, IN). The results of this procedure are traceable to the IDMS reference method and allow for accurate estimated glomerular filtration rate (eGFR). The reportable range of creatinine in serum/plasma samples is: 0.03–60.0 mg/dL, and 0.03–1200.0 mg/dL in urine samples. The immunochemical measurement of albumin in urine was performed by using Dade Behring reagent on a Behring Nephelometer (BNII) and use of the reference material CRM470 prepared by the International Federation of Clinical Chemistry (IFCC).

Islet cell autoantibody assays for IA-2 and GAD-65 were initially performed at the TODAY central laboratory at the University of Washington (GAD index and IA2 index). They were subsequently confirmed at the Diabetes Research Institute Munich Laboratory (Munich, Germany) (GAD-DK and IA2 DK) using the NIDDK standardized assay with standardized ^{35}S -labeled GAD-65 or IA2-IC proteins, according to the harmonized NIDDK/NIH autoantibody methods. The assays were calibrated using a set of standards with predetermined levels of GAD-65 or IA-2 antibodies expressed in arbitrary NIDDK units (DK units per ml). The GAD-65 assay is 76% sensitive and 97% specific, and the IA-2 assay is 64% sensitive and 99% specific. IA2 was defined to be positive if IA2-DK \geq 5; if IA2 DK was missing, IA2 index \geq 0.017 was considered evidence for positivity. GAD was defined to be positive if GAD Index \geq 0.085 or GAD-DK \geq 33.

ZnT8 antibodies were measured in the autoantibody laboratory at the Barbara Davis Center (Denver, Colorado) from stored samples at baseline, end of study, and at time of loss of glycemic control, if applicable. The samples had never previously been thawed. ZnT8

autoantibody (ZnT8A) radioassay was performed as described previously [2]. The ZnT8 protein was produced by in vitro transcription and translation (Promega TNT kit) and labeled with [³⁵S]methionine (Perkin Elmer). Labeled [³⁵S]ZnT8 was mixed and incubated overnight with patient serum and autoantibody-bound antigen precipitated with protein A-Sepharose (GE HealthCare) and washed. After washing, scintillation fluid, MicroScint-20 (Perkin Elmer), was added and radioactivity was counted on a TopCount 96-well plate counter (Perkin Elmer). The standard internal high and low, positive and negative control sera were included in every assay. The results are expressed as index (index = (sample CPM – negative control CPM)/(positive control CPM – negative control CPM). The upper limit of normal (0.020) was established as the 99th percentile from receiver operating characteristic curves in 100 healthy control subjects and 50 patients with new-onset diabetes. The sensitivity and specificity were 68 and 100%, respectively.

Statistical methods

Due to low ZnT8A positivity in this study, data reported are limited to descriptive statistics of the participants at the time of screening and whether or not they met the criteria for loss of glycemic control during their participation in TODAY. When appropriate, data are reported as mean ± SD or percentage within a category. The descriptive statistics, means and standard deviations for continuous variables and frequency and percent for discrete variables, are presented for data collected at baseline and at 24 months post randomization since this was the minimum duration of planned follow-up. We also present the rate of having the primary outcome, time to outcome, and rates of comorbidities for the full duration of the study. The Statistical Analysis Software package (SAS, version 9.4, SAS Institute Inc., Cary, NC) was used for all analyses. No adjustment was made for multiple comparisons.

Results

Of the 699 TODAY participants, 16 participants (3.0%) converted from negative to positive for GAD65 (GAD65+) and 3 (0.55%) for IA-2 during the study. Participants who converted to IA-2 positivity during the trial had higher insulin sensitivity and IL-1β concentration as well as a higher rate of loss of glycemic control compared to the whole TODAY cohort and IA-2 negative individuals (Table 1). For GAD, those who converted exhibited higher HbA1c, triglycerides, urine albumin/creatinine ratio, and more rapid loss of glycemic control than any other group (Table 1). These differences were not associated with any difference in insulin sensitivity, insulin secretion, or disposition index.

ZnT8 antibody results were available for 687 participants (99%). Only 2 of these (0.29%) were positive for ZnT8 antibodies (ZnT8+) at

screening and there was one conversion from ZnT8- to ZnT8 + during the study. Of the 3 participants with ZnT8 positivity, none were ever found to be GAD positive but one participant, who had ZnT8 positivity at baseline, also had IA-2 positivity post-baseline. There was no difference in any parameter measured between individuals who had ever been ZnT8A + or those who had repeated ZnT8A+, therefore the groups were combined (Table 2). ZnT8A + individuals had higher HbA1c, HDL and LDL cholesterol, and IL-1β concentrations, and lower BMI, IL-6, and triglyceride concentrations compared to both the entire TODAY cohort and ZnT8A negative individuals. They also had higher insulin sensitivity with lower insulin secretion, and a lower disposition index. Of note, 100% of ZnT8 + participants reached the study primary outcome of loss of glycemic control compared to 45% of ZnT8 negative participants. In addition, ZnT8 + participants lost glycemic control more rapidly than other groups (Table 2).

Discussion

Autoantibodies in the TODAY cohort

Given the very low number of cases, all differences noted herein are observational and lack power for analysis of statistical significance. Nevertheless, this is the largest group of adolescents with phenotypic type 2 diabetes who have been systematically screened for antibodies and followed for an extended period to identify clinical trajectory.

In the TODAY cohort, a very small number of participants converted to GAD+ (3%) or IA-2+ (0.55%). This argues against autoimmunity – either missed at diagnosis or developing secondarily - being an important aspect of youth-onset type 2 diabetes. However, those individuals who did transition to antibody-positivity, like those previously reported to be positive at screening [1], had a phenotype closer to that of T1D (demographics, rapid loss of glycemic control on T2D therapy, higher insulin sensitivity, lower insulin secretion, lower disposition index etc) than those who remained antibody-negative. Although these are small numbers, these findings suggest that repeat antibody testing may be indicated in those individuals with rapid loss of glycemic control to help determine the appropriateness of added agents (insulin vs. intensified T2D therapy). However, this approach may not be cost effectiveness given the low rate of conversion and the known rapid deterioration of beta-cell function in youth-onset T2D who are antibody-negative.

ZnT8 + and its associated phenotype

There was a very low prevalence of ZnT8+ (0.43%) in our cohort of adolescents with T2D, in contrast to the reported 26% prevalence in T1D antibody-negative children overall (4). This indicates that ZnT8

Table 1
Results at 24 Months and TODAY Outcome and Comorbidities by Overall TODAT cohort, IA2 Status and GAD Status.

	Overall (N = 548)		IA2 Positive		GAD Positive	
	Mean ± SD or N and %		No (N = 545)	Yes (N = 3)	No (N = 532)	Yes (N = 16)
BMI percentile	96.90 ± 5.36		96.98 ± 5.03	83.56 ± 26.15	96.89 ± 5.39	97.29 ± 4.18
HbA1c (%)	7.4 ± 2.4		7.4 ± 2.4	7.5 ± 0.5	7.4 ± 2.4	7.6 ± 2.0
HbA1c (mmol/mol)	57 ± 26.2		57 ± 26.2	58 ± 5.5	57 ± 26.2	60 ± 21.9
Total Cholesterol (mg/dL)	157.10 ± 36.63		157.21 ± 36.67	137.67 ± 25.77	157.11 ± 36.89	156.81 ± 27.82
HDL Cholesterol (mg/dL)	41.00 ± 10.10		41.02 ± 10.12	37.67 ± 7.37	40.99 ± 10.17	41.38 ± 8.16
LDL Cholesterol (mg/dL)	90.00 ± 28.49		90.07 ± 28.51	77.00 ± 27.18	89.94 ± 28.67	91.88 ± 22.60
Triglycerides (mg/dL)	133.23 ± 139.02		133.33 ± 139.38	115.33 ± 48.69	133.71 ± 140.66	117.56 ± 67.78
Urine Albumin/Creatinine Ratio	35.75 ± 126.99		35.73 ± 127.24	40.50 ± 38.89	35.45 ± 127.86	44.81 ± 100.32
Insulin Inverse [1/I ₀] (mL/uU)	0.05 ± 0.04		0.05 ± 0.04	0.09 ± 0.10	0.05 ± 0.04	0.04 ± 0.02
C-peptide Index [ΔC ₃₀ /ΔG ₃₀] (ng/mL per mg/dL)	0.06 ± 0.07		0.06 ± 0.07	0.01 ± 0.01	0.06 ± 0.07	0.06 ± 0.08
coDI [1/IF × ΔC ₃₀ /ΔG ₃₀]	0.00285 ± 0.0037		0.00286 ± 0.0037	0.00005 ± 0.0000	0.00285 ± 0.0037	0.00283 ± 0.0021
IL-1β (pg/mL)	1.05 ± 1.47		1.03 ± 1.46	3.16 ± 1.74	1.05 ± 1.49	1.06 ± 0.88
IL-6 (pg/mL)	2.35 ± 1.78		2.34 ± 1.77	3.17 ± 2.64	2.31 ± 1.71	3.65 ± 3.13
Had primary outcome*	301 50.8%		299 50.8%	2 66.7%	291 50.6%	10 58.8%
Time to primary outcome (days)*	972.99 ± 669.55		972.50 ± 669.36	1068.6 ± 857.25	979.55 ± 666.67	751.00 ± 748.75

Table 2
Demographic Characteristics and Results at Baseline and 24 Months Overall TODAY Cohort and by ZnT8 Status.

	Overall (N = 692)		ZnT8 Ever Positive			
			No (N = 688)		Yes (N = 3)	
	Mean ± SD or N and %		Mean ± SD or N and %		Mean ± SD or N and %	
Baseline age (years)	13.98	± 2.03	13.98	± 2.03	14.00	± 2.00
Months since diagnosis at baseline	7.77	± 5.80	7.76	± 5.78	10.00	± 9.54
Female	445	64.4%	443	64.4%	2	66.7%
Race/Ethnicity						
Non-Hispanic Black	224	32.4%	223	32.4%	1	33.3%
Hispanic	276	39.9%	276	40.1%	0	0%
Non-Hispanic White	140	20.3%	138	20.1%	2	66.7%
Other	51	7.4%	51	7.4%	0	0%
Positive family history of diabetes	401	58.0%	400	58.1%	1	33.3%
BMI Percentile						
Baseline	97.69	± 3.32	97.75	± 3.13	83.68	± 12.23
24 months	96.89	± 5.53	97.00	± 5.20	75.89	± 20.14
HbA1c (%)						
Baseline	6.0	± 0.7	6.0	± 0.7	6.0	± 0.8
24 months	7.3	± 2.4	7.3	± 2.4	9.6	± 2.7
HbA1c (mmol/mol)						
Baseline	42	± 7.7	42	± 7.7	42	± 8.7
24 months	56	± 26.2	56	± 26.2	81	± 29.5
Total Cholesterol (mg/dL)						
Baseline	145.92	± 29.35	145.91	± 29.40	149.33	± 13.87
24 months	156.71	± 36.56	156.67	± 36.60	164.67	± 32.58
HDL Cholesterol (mg/dL)						
Baseline	38.66	± 8.57	38.63	± 8.57	46.33	± 4.51
24 months	41.16	± 10.41	41.11	± 10.41	50.33	± 5.13
LDL Cholesterol (mg/dL)						
Baseline	84.87	± 24.64	84.84	± 24.68	90.33	± 8.62
24 months	89.86	± 28.33	89.81	± 28.36	100.33	± 23.29
Triglycerides (mg/dL)						
Baseline	114.46	± 78.31	114.68	± 78.40	63.67	± 18.50
24 months	131.05	± 134.78	131.36	± 135.05	71.67	± 22.03
Urine Albumin/Creatinine Ratio						
Baseline	32.02	± 124.13	32.05	± 124.23	11.00	± 0
24 months	41.51	± 186.39	41.55	± 186.56	16.00	–
Insulin Inverse [1/10] (mL/uU)						
Baseline	0.05	± 0.04	0.05	± 0.04	0.12	± 0.07
24 months	0.05	± 0.04	0.05	± 0.04	0.16	–
C-peptide Index [ΔC30/ΔG30] (ng/mL per mg/dL)						
Baseline	0.08	± 0.12	0.08	± 0.12	0.02	± 0.02
24 months	0.06	± 0.07	0.06	± 0.07	0.03	± 0.05
coDI [1/IF × ΔC30/ΔG30]						
Baseline	0.00342	± 0.0059	0.00342	± 0.0059	0.00245	± 0.0015
24 months	0.00290	± 0.0037	0.00291	± 0.0037	0.00002	–
IL-1β (pg/mL)						
Baseline	1.07	± 1.01	1.05	± 0.95	8.96	–
24 months	1.05	± 1.46	1.04	± 1.45	3.03	± 2.64
IL-6 (pg/mL)						
Baseline	2.23	± 1.70	2.23	± 1.70	1.01	± 0.53
24 months	2.35	± 1.79	2.35	± 1.80	1.45	± 1.45
Had primary outcome	315	45.6%	312	45.3%	3	100.0%
Time to primary outcome (days)	487.57	± 450.37	488.85	± 451.45	354.00	± 354.51

autoantibody positivity is not the etiology of a significant number of cases of T2D in youth, at least when those with other antibody-positivity are excluded from testing. Although ZnT8 + incidence is very low in the TODAY cohort, it is associated with higher insulin sensitivity, lower insulin secretion, and lower disposition index, i.e., a pattern more closely resembling the characteristics for T1D [5] and of those positive for GAD and IA-2 at screening [1]. These findings support the notion that TODAY ZnT8 + participants have a phenotype consistent with autoimmune diabetes. Therefore, a very small number of youth with diabetes in the setting of obesity could be mischaracterized as T2D if ZnT8 testing is not included in antibody screening. However, given the very low prevalence of ZnT8 positivity in individuals with phenotypic T2D who are otherwise antibody-negative, the cost-effectiveness of routine ZnT8 testing is unclear.

Individuals who were ZnT8 + at any time had 100% loss of glycemic control on randomized T2D therapies without insulin. For GAD and IA-2, the risk of loss of glycemic control was slightly increased in those who ever had a positive test but was 100% in the small number of participants who had repeated positive values. Furthermore, the time to loss of glycemic control was shorter in all participants who were ever found to have positive antibodies.

Study strengths and limitations

Despite the small numbers of identified participants with positive antibodies, the strengths of this study are that this is the largest cohort of children with T2D and systematic antibody testing using gold-standard assays and extended clinical follow-up with a standardized protocol and

central laboratory, allowing for rigorous understanding of the trajectory of outcome in antibody-positive individuals. Limitations include the lack of testing for insulin autoantibodies at screening and subsequently due to the prior exposure of individuals to insulin, therefore, we cannot exclude the possibility that some or all of the ZnT8 positive patients or those who converted to IA2 or GAD antibody positivity also have insulin autoantibodies and were not, therefore, otherwise antibody negative.

Conclusion

The prevalence of ZnT8 positivity in youth with phenotypic T2D is very low, as is the conversion from GAD and IA2 antibody-negativity to antibody-positivity over 2 years. However, the presence of antibodies at any point in the clinical course is associated with higher insulin sensitivity, lower insulin secretion, and lower disposition index, suggesting a more T1D-like phenotype. Most importantly, however, antibody positivity at any point in the clinical course is associated with a higher rate of and shorter time to loss of glycemic control on oral anti-hyperglycemic therapy. These results suggest that repeat antibody testing might be indicated for those with rapid loss of glycemic control to help determine the best choices for intensification of therapy. However, given the low rates of antibody conversion and low prevalence of ZnT8 antibody, routine screening for ZnT8 and repeat testing for GAD and IA-2 autoantibodies may not be cost-effective.

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