



Hierarchical Order of Distinct Autoantibody Spreading and Progression to Type 1 Diabetes in the TEDDY Study

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OBJECTIVE

The first-appearing β -cell autoantibody has been shown to influence risk of type 1 diabetes (T1D). Here, we assessed the risk of autoantibody spreading to the second-appearing autoantibody and further progression to clinical disease in The Environmental Determinants of Diabetes in the Young (TEDDY) study.

RESEARCH DESIGN AND METHODS

Eligible children with increased HLA-DR-DQ genetic risk for T1D were followed quarterly from age 3 months up to 15 years for development of a single first-appearing autoantibody (GAD antibody [GADA], insulin autoantibody [IAA], or insulinoma antigen-2 autoantibody [IA-2A]) and subsequent development of a single second-appearing autoantibody and progression to T1D. Autoantibody positivity was defined as positivity for a specific autoantibody at two consecutive visits confirmed in two laboratories. Zinc transporter 8 autoantibody (ZnT8A) was measured in children who developed another autoantibody.

RESULTS

There were 608 children who developed a single first-appearing autoantibody (IAA, $n = 282$, or GADA, $n = 326$) with a median follow-up of 12.5 years from birth. The risk of a second-appearing autoantibody was independent of GADA versus IAA as a first-appearing autoantibody (adjusted hazard ratio [HR] 1.12; 95% CI 0.88–1.42; $P = 0.36$). Second-appearing GADA, IAA, IA-2A, or ZnT8A conferred an increased risk of T1D compared with children who remained positive for a single autoantibody, e.g., IAA or GADA second (adjusted HR 6.44; 95% CI 3.78–10.98), IA-2A second (adjusted HR 16.33; 95% CI 9.10–29.29; $P < 0.0001$), or ZnT8A second (adjusted HR 5.35; 95% CI 2.61–10.95; $P < 0.0001$). In children who developed a distinct second autoantibody, IA-2A (adjusted HR 3.08; 95% CI 2.04–4.65; $P < 0.0001$) conferred a greater risk of progression to T1D as compared with GADA or IAA. Additionally, both a younger initial age at seroconversion and shorter time to the development of the second-appearing autoantibody increased the risk for T1D.

CONCLUSIONS

The hierarchical order of distinct autoantibody spreading was independent of the first-appearing autoantibody type and was age-dependent and augmented the risk of progression to T1D.

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*A complete list of the TEDDY Study Group members is provided in the supplementary material online.

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β -cell autoantibodies are predictors of type 1 diabetes (T1D) (1,2) and are currently the most reliable indicator of preclinical disease. The presence of two or more autoantibodies against the four major autoantigens (insulin, GAD, insulinoma antigen-2 [IA-2], and zinc transporter 8 [ZnT8]) have been shown to confer the highest risk of T1D and was recently defined as stage 1 T1D (3). β -cell autoantibodies develop prior to and usually persist over time in the progression to clinical onset of T1D (stage 3). The age at appearance of specific β -cell autoantibodies varies (4–6). GAD antibody (GADA) or insulin autoantibody (IAA) predominate as first-appearing autoantibodies (7,8). IAA appearance peaks within the first 2 years of life. In contrast, GADA appears between ages 3 and 5 years in children with either increased genetic risk or family history of T1D (7–9). IA-2 autoantibody (IA-2A) and ZnT8 autoantibody (ZnT8A) generally appear after the initial islet autoantibody seroconversion (6,8,10,11) in more advanced stages of the disease process (3,12–14).

Recent studies have focused on the appearance of the first-appearing autoantibody (7,8) with limited discussion of the specific second-appearing autoantibody (8). The Finnish T1D Prediction and Prevention (DIPP) study (8) reported that IAA as a second-appearing autoantibody peaked in the first 5 years of life and followed a similar pattern to the first-appearing GADA, whereas GADA peaked within the first 2 years of life and mostly occurred after IAA. More recently, the DIPP study reported that the initial age at seroconversion was only associated with IAA-initiated seroconversion and further progression to T1D (15). However, because of a varied autoantibody screening interval, further exploration after the first 2 years of life was limited. Other studies assessing the presence of autoimmunity and combinations of autoantibodies were carried out where the actual order of appearance could not be distinguished because of annual screening (16,17) or capture was at the time of or just prior to diagnosis (1,11,18–22). These previous studies assessing risk from single to multiple autoantibodies showed that increased risk was associated with a younger age, HLA-DR-DQ, and IAA as the first-appearing autoantibody (16,17). In addition to HLA class II risk, a

recent study from the Belgian Diabetes Registry (23) reported that specific HLA class I alleles accelerated progression from multiple autoantibody positivity to T1D in those <40 years with a family history of T1D. Specifically, HLA-A*24 was associated with an accelerated progression in HLA-DQ8⁺ relatives who developed either IA-2A or ZnT8A, whereas HLA-B*18 was associated with accelerated progression in HLA-DQ2⁺ relatives who developed both IAA and GADA (23). Moreover, non-HLA single nucleotide polymorphisms (SNPs), such as those in the *INS-VNTR* gene region (rs1004446) and protein tyrosine phosphatase non-receptor type 22 (*PTPN22*, rs2476601) have been shown to associate with a decreased and increased risk of progression to islet autoimmunity and T1D, respectively (24,25). Thus, understanding the order and time of appearance of each distinct autoantibody will allow for better stratification of risk.

The aims of this study were 1) to assess the risk of developing a distinct second islet autoantibody in children at increased genetic risk for T1D who newly seroconverted for either IAA-only or GADA-only first-appearing autoantibodies and 2) to determine the risk associated with the specific target of the first- and second-appearing autoantibody combination on further progression to clinical T1D.

RESEARCH DESIGN AND METHODS

Study Population

The Environmental Determinants of Diabetes in the Young (TEDDY) is a prospective cohort study of children at an increased genetic risk for T1D funded by the National Institutes of Health. The TEDDY study seeks to identify environmental causes of T1D. There are six clinical research centers: three in the U.S. (Colorado, Georgia/Florida, and Washington) and three in Europe (Finland, Germany, and Sweden). The high-risk genotypes for subjects screened from the general population with no family history of T1D (89%) were as follows: DRB1*04-DQA1*03-DQB1*03:02/DRB1*03-DQA1*05-DQB1*02:01 (DR3/4-Q2/8), DRB1*04-DQA1*03-DQB1*03:02/DRB1*04-DQA1*03-DQB1*03:02 (DR4/4-DQ8/8), DRB1*04-DQA1*03-DQB1*03:02/DRB1*08-DQA1*04-DQB1*04:02 (DR4/8-DQ8/4), and DRB1*03-DQA1*05-DQB1*02:01/DRB1*03-DQA1*05-DQB1*02:01 (DR3/3-DQ2/2) and six additional genotypes in first-degree relatives (FDRs) of those

with a family history of T1D as previously described (26). There were 8,676 children enrolled from September 2004 to February 2010 and currently followed prospectively from age 3 months to 15 years with study visits every 3 months until 4 years and every 3 or 6 months, thereafter, depending on autoantibody positivity. All children who are persistently positive for any autoantibody are followed every 3 months until the age of 15 years or onset of T1D. Detailed study design and methods have been published previously (26–28). The protocol was approved by institutional review boards at participating centers, and all participants provided written informed consent before participation in the genetic screening and enrollment.

β -Cell Autoantibodies

β -cell autoantibodies to IAA, GADA, or IA-2A were measured in two laboratories by radiobinding assays (27,29). In the U.S., all sera were assayed at the Barbara Davis Center for Childhood Diabetes at the University of Colorado Denver; in Europe, all sera were assayed at the University of Bristol, in the U.K. Both laboratories reported high sensitivity, specificity, and concordance (29,30). All positive β -cell autoantibodies and 5% of negative samples were retested in the other reference laboratory and deemed confirmed if concordant. Autoantibody positivity (persistent confirmed) was defined as a specific autoantibody presence on greater than or equal to two consecutive visits 3 months apart and confirmed in two TEDDY laboratories. The date of positivity was the draw date of the first sample of the two consecutive positive samples that deemed a child persistent confirmed positive for a specific autoantibody. The age of seroconversion was the age of the child on the initial date of seroconversion to the first persistent confirmed autoantibody. The age of second autoantibody seroconversion was the age of the child on the date of autoantibody seroconversion of a second persistent confirmed autoantibody. Stage 1 T1D was defined as the presence of multiple autoantibodies (3). Symptomatic T1D (stage 3) was defined according to American Diabetes Association criteria for diagnosis (3,31). There were 8,502 of the 8,676 children eligible for this analysis; 174 were ineligible for this analysis and excluded due to not having

an autoantibody test result ($n = 54$) or were deemed HLA-DR-DQ ineligible based on study criteria ($n = 120$) (Supplementary Fig. 1 and Supplementary Table 1). As the focus of this analysis was to examine autoantibody spreading, the analyses were restricted to the development of the first-appearing IAA single ($n = 282$) or GADA single ($n = 326$) as of 31 December 2018 and followed through 31 December 2019, excluding children who developed multiple autoantibodies (greater than or equal to two autoantibodies) at their initial seroconversion ($n = 156$) or IA-2A only ($n = 15$), or ZnT8A only ($n = 12$). There were 608 children included in these analyses.

Zinc Transporter 8 Autoantibodies

ZnT8A radioimmunoassay was performed as previously described (19). The ZnT8 protein was produced by in vitro transcription and translation (TNT kit; Promega, Madison, WI) and labeled with [35S]methionine (Perkin Elmer, Waltham, MA). [35S]ZnT8 (20,000 cpm) was mixed and incubated overnight at 4°C with 2- μ L serum in an assay buffer (20 mmol/L Tris HCl, pH 7.4, supplemented with 150 mmol/L NaCl, 0.1% [w/v] BSA, 0.15% [v/v] Tween-20 and 0.1% [w/v] sodium azide) at a final 1:25 dilution. Autoantibody-bound antigen was precipitated with 25- μ L 50% protein A-Sepharose (GE HealthCare, Piscataway, NJ) in an opaque 96-well filtration plate (Corning, Tewksbury, MA) and washed with two cycles of four washes per each cycle of cold assay buffer using a vacuum-operated 96-well plate washer (BioTek, Winooski, VT). After washing, scintillation fluid, MicroScint-20 (Perkin Elmer), was added directly to the 96-well plate, and radioactivity was counted on a Top-Count 96-well plate β -counter (Perkin Elmer). The results are expressed as an 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 the receiver operating characteristic curves in 100 healthy control subjects and 50 patients with new-onset diabetes. In the most recent Islet Autoantibody Standardization Program (2017) workshop, the sensitivity and specificity were 66 and 100%, respectively. ZnT8A was run on all available samples at the TEDDY Barbara Davis Laboratory, if a child was deemed

antibody positive for IAA, GADA, and/or IA-2A (any number of autoantibodies, any autoantibody, persistence or confirmation not required) at a visit. Persistent ZnT8A was defined as the presence of ZnT8A on greater than or equal to two consecutive visits 3 months apart in a single laboratory. Protocol compliance for measurement of ZnT8A prior to the first autoantibody positive sample in children with persistent IAA, IA-2A, or GADA was 99%. For children who did not meet protocol compliance ($n = 9$), the median months following the first autoantibody positive sample was 3.7 months.

Statistical Analysis

Cumulative incidence of the appearance of the second autoantibody and the risk of progression to T1D were examined in Kaplan-Meier analyses. Multivariable proportional hazards (PH) models were applied to assess both the cause-specific risk of autoantibody spreading to a second-appearing autoantibody and the risk of progression to T1D from the second-appearing autoantibody. Each specific second-appearing autoantibody was also examined as a time-varying predictor on risk of T1D. To ensure that the second-appearing autoantibody was compared with single autoantibody-positive children in each risk set, the other single second-appearing autoantibodies or multiple second-appearing autoantibodies (two autoantibodies appearing during the same 3-month interval) were censored at the time of their appearance as defined above. The strength of the associations with T1D was described by hazard ratios (HRs) and with second-appearing autoantibodies by cause-specific hazard ratios (CHRs) with 95% CIs. All multivariable analyses were adjusted for HLA-DR-DQ, sex, family history of T1D, age at initial seroconversion, and first-appearing autoantibody (IAA or GADA). The following also considered for adjustment (and remained in the final model if $P < 0.10$) were HLA class I alleles (HLA-A*24:X, -B*18:X:Y) (23), *SLC30A8* (rs13266634) (32), SNPs previously shown to be associated with islet autoimmunity and T1D in TEDDY (*INS* [rs1004446], *PTPN22* [rs2476601], *ERBB3* [rs2292239], *SH2B3* [rs3184504], *TNFAIP3* [rs2327832], rs1534422, and rs10517086) (5,24,25,33) and characteristics found to be associated with islet autoimmunity and T1D (34–37), unless otherwise stated. Country of

residence was included in all models as a stratified variable. Adjustments for population stratification were made by using the top two principal components from the Immunochip SNP data as covariates in the PH model (38). Data were analyzed using the SAS Software (version 9.4; SAS Institute, Cary, NC) and GraphPad PRISM 7.04 (GraphPad Software Inc., San Diego, CA) for figures. Two-tailed P values < 0.05 were considered significant.

RESULTS

As of 31 December 2018, 608 of the 8,502 enrolled children with HLA-DR-DQ genetic risk for T1D in TEDDY developed a single positive IAA or GADA at their initial seroconversion and met the inclusion criteria for this analysis: IAA only ($n = 282$) and GADA only ($n = 326$). Of those who developed first-appearing IAA or GADA only ($n = 608$), 272 (44.7%) remained positive for a single autoantibody for the duration of follow-up through 31 December 2019, and 10.3% ($n = 28/272$) of these single autoantibody-positive children progressed to T1D within 0.95 (0.28–3.37) years; 55.3% ($n = 336/608$) developed another autoantibody, and 53.0% ($n = 178/336$) of these children progressed to T1D within 3.53 (1.56–5.83) years. The median (interquartile range [IQR]) age at initial GADA- or IAA-only seroconversion was 4.28 (2.27–7.51) and 1.83 (1.00–3.82) years, respectively. The median (IQR) follow-up from the initial seroconversion was 5.7 (3.2–8.7) years with the majority (98%) followed for ≥ 2 years.

Time From First- to Second-Appearing Autoantibody

The characteristics of the children by specific second-appearing autoantibody are shown in Supplementary Table 2, and the age-descriptive patterns of the second-appearing autoantibody are shown in Supplementary Fig. 2. GADA as a second-appearing autoantibody developed at a median (IQR) of 3.7 (3.0–9.7) months after the first-appearing autoantibody; IAA as a second-appearing autoantibody developed at a median of 5.9 (3.0–25.9) months; IA-2A as a second-appearing autoantibody developed at a median of 6.9 (3.7–17.6) months; and ZnT8A as a second-appearing autoantibody developed at a median of 13.3 (6.3–23.7) months after the first-appearing autoantibody. The overall median

(Q1-Q3) time after the first-appearing autoantibody to the appearance of the second autoantibody was 6.8 (3.2–17.0) months. There was no significant difference in the time from the first- to second-appearing autoantibody by the type of first-appearing IAA or GADA, taking into account the age at the initial seroconversion.

Risk Factors Associated With the Development of a Second-Appearing Autoantibody

Known T1D risk factors were examined for univariate association with developing a second autoantibody (Supplementary Table 3). Family history of T1D was positively associated with the development of a second autoantibody, whereas having a minor allele in the *INS-VNTR* gene region (rs1004446) or HLA-DR3/3 was inversely associated with developing IAA or GADA as a second autoantibody. The age at initial seroconversion was inversely associated with the development of a second autoantibody in the multivariable analyses (Table 1). Of interest, the risk of developing a second autoantibody declined with increasing age at initial seroconversion (for each additional month older, adjusted HR 0.986; 95% CI 0.983–0.990; $P < 0.0001$).

From the time of the first-appearing autoantibody, the risk of appearance of any second autoantibody did not differ if GADA or IAA was the first-appearing autoantibody (adjusted HR 1.12; 95%

CI 0.88–1.42; $P = 0.36$), independent of age at initial seroconversion. Further assessment did not find any specific second autoantibody associated with a specific first-appearing autoantibody (IAA or GADA) after the Bonferroni correction for significance at $P < 0.008$ for multiple comparisons (Fig. 1A and B). Overall, there was no significant difference based on either GADA only or IAA only as the first-appearing autoantibody on the risk of developing IA-2A (adjusted CHR 1.23; 95% CI 0.69–2.20; $P = 0.48$) or ZnT8A (adjusted CHR 1.62; 95% CI 0.91–2.86; $P = 0.09$) as a second-appearing autoantibody.

Influence of a Second Autoantibody on Risk of Progression to T1D

We next asked if the appearance of the second autoantibody (time-varying predictor) from the time of the first-appearing autoantibody influenced progression to T1D. Children who developed a distinct second-appearing autoantibody had an increased risk of progression to T1D as compared with children who remained positive for a single autoantibody for IAA or GADA second (adjusted HR 6.44; 95% CI 3.78–10.98; $P < 0.0001$), IA-2A second (adjusted HR 16.33; 95% CI 9.10–29.29; $P < 0.0001$), and ZnT8A second (adjusted HR 5.35; 95% CI 2.61–10.95; $P < 0.0001$) from age of first-appearing autoantibody, adjusting for age at initial seroconversion, sex, family

history of T1D, HLA-DR-DQ, *INS-VNTR* gene region (rs1004446), and type of first-appearing autoantibody. No other HLA class I alleles or non-HLA SNPs assessed were found to significantly associate with developing a specific second autoantibody.

Risk Factors Associated With T1D in Children With Multiple Autoantibodies

In children who developed multiple autoantibodies, IA-2A, as a second-appearing autoantibody, conferred a significantly greater risk of progression to T1D (adjusted HR 3.08; 95% CI 2.04–4.65; $P < 0.0001$) (Table 2 and Fig. 2) as compared with IAA or GADA. There was no statistical evidence that the appearance of ZnT8A as a second autoantibody as compared with IAA or GADA modified risk of progression to T1D (adjusted HR 0.81; 95% CI 0.51–1.29; $P = 0.37$). Other covariates significantly associated with progression in children who developed multiple autoantibodies were age at the appearance of the first autoantibody (adjusted HR 0.98; 95% CI 0.97–0.99; $P = 0.001$), time in months from the first to second autoantibody (adjusted HR 0.97; 95% CI 0.96–0.99; $P = 0.003$), and sex of the child (female versus male, adjusted HR 1.52; 95% CI 1.09–2.13; $P = 0.014$). Further assessment of specific HLA class I alleles and non-HLA SNPs (including interactions) previously shown to be associated with T1D were not found

Table 1—Multivariable CHRs in children with a single first-appearing IAA or GADA positivity ($n = 608$) for progression to second autoantibody positivity ($n = 336\ddagger$)

	Second-appearing autoantibody					
	GADA or IAA		IA-2A		ZnT8A	
	CHR* (95% CI)	<i>P</i> value	CHR* (95% CI)	<i>P</i> value	CHR* (95% CI)	<i>P</i> value
Female vs. male	0.90 (0.65–1.23)	0.50	1.12 (0.67–1.87)	0.67	0.64 (0.38–1.07)	0.09
Family history of T1D (reference = no family history)						
Sibling with T1D	2.22 (1.12–4.40)	0.023	0.86 (0.12–6.43)	0.89	1.93 (0.57–6.48)	0.29
Father with T1D	1.35 (0.82–2.23)	0.24	1.54 (0.71–3.35)	0.27	2.15 (1.04–4.46)	0.039
Mother with T1D	0.80 (0.31–2.10)	0.65	2.88 (1.06–7.84)	0.039	2.15 (0.66–7.08)	0.21
HLA-DR-DQ genotype (reference = DR4/4)						
DR3/4	1.26 (0.83–1.93)	0.28	1.21 (0.58–2.52)	0.62	1.20 (0.56–2.54)	0.64
DR4/8	0.55 (0.29–1.01)	0.05	1.74 (0.76–4.01)	0.19	1.01 (0.40–2.57)	0.98
DR3/3	0.32 (0.16–0.67)	0.003	0.69 (0.26–1.85)	0.46	0.96 (0.40–2.30)	0.93
FDR-specific**	1.01 (0.35–2.89)	0.98	0.38 (0.04–3.30)	0.38	NA	0.98
SNP rs1004446_A (<i>INS</i>)	0.70 (0.51–0.96)	0.025	0.94 (0.56–1.57)	0.81	1.03 (0.62–1.72)	0.90
First-appearing Ab (GADA vs. IAA)	0.94 (0.67–1.33)	0.73	1.23 (0.69–2.20)	0.48	1.62 (0.91–2.86)	0.09
Age at first-appearing autoantibody	0.99 (0.98–0.99)	<0.0001	0.98 (0.97–0.99)	0.0002	0.99 (0.98–99)	0.003

Boldface type indicates statistical significance. NA, not applicable. *Stratified Cox regression with country of residence. **FDR-specific HLA-DR-DQ genotypes are the following: DR4/1, DR4/9, DR4/13, DR3/9, DR4/4-DQB1*20X, and DR4/4-DQB1*304. \ddagger 47 children developed two autoantibodies within the same 3-month interval as second-appearing autoantibodies and were censored at time of second-appearing autoantibody positivity. Data were not presented due to difficulty in determining order of appearance.

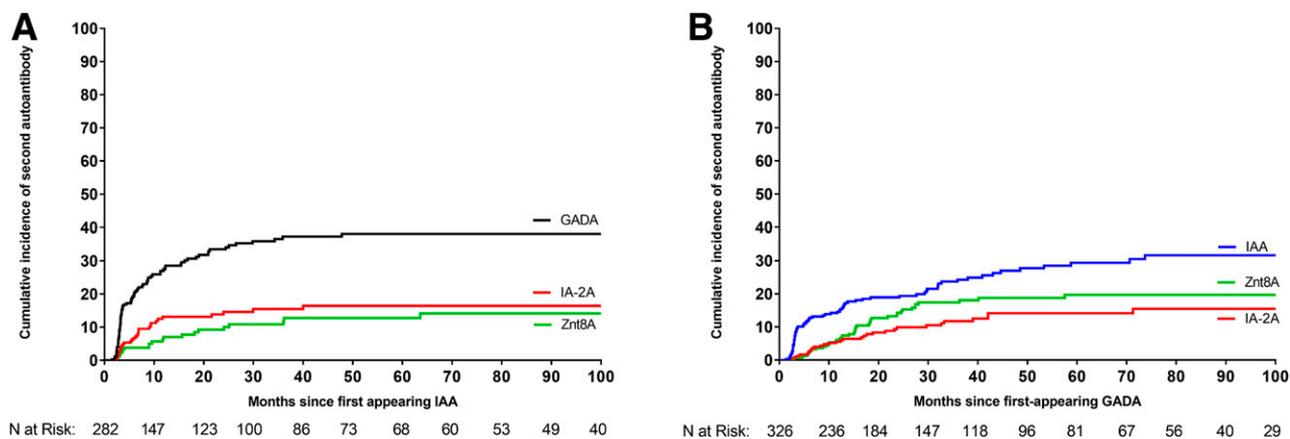


Figure 1—Cumulative incidence in children with distinct single first-appearing autoantibody positivity (IAA or GADA) for progression to distinct single second-appearing autoantibody positivity. *A*: The cumulative incidence of the second-appearing autoantibody by months since first-appearing IAA. *B*: The cumulative incidence of the second-appearing autoantibody by months since first-appearing GADA. The lines represent IAA (blue line), GADA (black line), IA-2A (red line), and ZnT8A (green line).

significantly associated with progression in this population with increased HLA-DR-DQ genetic risk.

Time Effect of First- to Second-Appearing Autoantibody on Risk of T1D

Finally, we examined if the time duration between the first and second autoantibody influenced the risk of progression to T1D after the development of the second autoantibody. The risk of

progression from the second-appearing autoantibody to T1D declined with increasing time duration in months between the first- and second-appearing autoantibody (for every additional month longer, adjusted HR 0.97; 95% CI 0.96–0.99; $P = 0.006$) (Table 2). Such that there was a twofold increased risk of progression to T1D in children who developed a second autoantibody within 1 year of their first-appearing autoantibody

as compared with those who developed it after 1 year after the initial seroconversion (adjusted HR 2.24; 95% CI 1.45–3.46; $P = 0.0003$).

CONCLUSIONS

In this large cohort of children at increased genetic risk for T1D with either IAA or GADA as a first-appearing autoantibody, we found that the appearance of a second autoantibody, regardless of type, conferred at least a fivefold increased risk of progression to T1D as compared with children who remained single autoantibody positive. This increased risk of progression varied by type of second-appearing autoantibody with IA-2A conferring the highest risk as compared with GADA, IAA or ZnT8A and was altered by how rapid the second autoantibody appeared. Development within 1 year doubled the risk of progression to T1D. Furthermore, a younger age at the initial seroconversion increased the risk of developing a second autoantibody, and the type of second-appearing autoantibody was independent of the type of the first-appearing autoantibody. Our results showed that having a family member with T1D at the time of screening (sibling, father, or mother) was associated with the risk of a specific second autoantibody. Also, carrying HLA-DR3/3 or the minor allele in rs1004446, a SNP in the *INS-VNTR* gene region, decreased the chance of IAA or GADA as a second-appearing autoantibody. Knowing the type of second autoantibody and how quickly it develops would increase our ability to identify

Table 2—Multivariable proportional HRs in children with a distinct second-appearing autoantibody ($n = 289$) for progression to T1D ($n = 149$)

	HR (95% CI)	<i>P</i> values
Type of second antibody		
IAA or GADA	1 (reference)	
IA-2A	3.08 (2.04–4.65)	<0.0001
ZnT8A	0.81 (0.51–1.29)	0.37
Age at first-appearing autoantibody (months)	0.98 (0.97–0.99)	0.001
Time from first to second autoantibody (months)	0.97 (0.96–0.99)	0.003
Sex		
Female	1.52 (1.09–2.13)	0.014
Male	1 (reference)	
Family history of T1D		
Mother with T1D	1.55 (0.65–3.67)	0.32
Father with T1D	1.01 (0.60–1.70)	0.97
Sibling with T1D	0.92 (0.46–1.76)	0.82
General population (no T1D history)	1 (reference)	
HLA-DR-DQ genotype		
DR3/4	1.70 (0.99–2.93)	0.06
DR4/4	1 (reference)	
DR4/8	1.32 (0.69–2.54)	0.41
DR3/3	0.97 (0.44–2.15)	0.94
FDR-specific*	1.28 (0.35–4.63)	0.71
Type of first antibody		
GADA only	0.97 (0.66–1.43)	0.89
IAA only	1 (reference)	

*FDR-specific HLA-DR-DQ genotypes are the following: DR4/4b, DR4/1, DR4/9, DR4/13, and DR3/9.

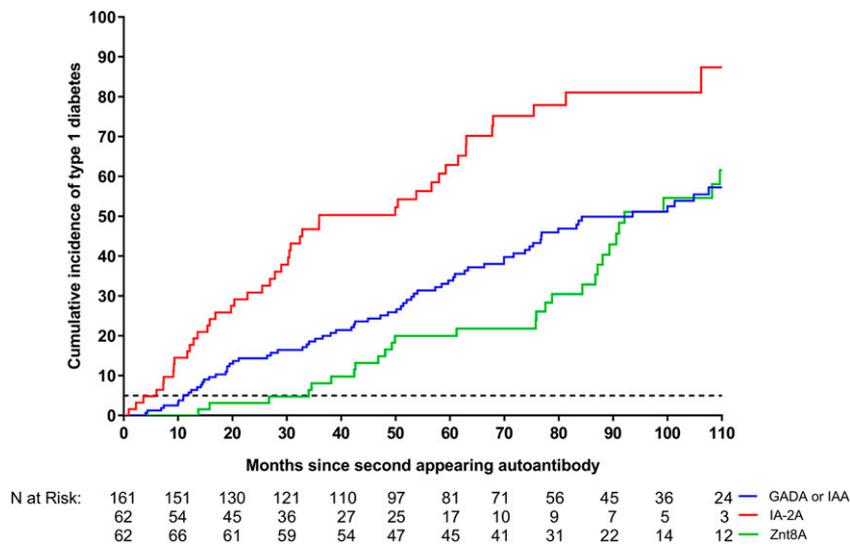


Figure 2—Cumulative incidence in children with a distinct single second-appearing autoantibody for progression to T1D: GADA or IAA (blue line), IA-2A (red line), and ZnT8A (green line). The 5-year (60-month) cumulative risk of T1D among the children who remained single autoantibody positive is shown (black dotted line). The median (IQR) time between the first- and second-appearing autoantibody seroconversions was 5.3 (3.0–13.3) months for GADA or IAA, 6.9 (3.7–17.6) months for IA-2A, and 13.3 (6.3–23.7) months for ZnT8A.

subjects who are more likely to have a rapid versus a slow progression to T1D.

The strengths of this study include the ability to assess the risk of the appearance of another autoantibody and the temporal impact of this second autoantibody after the time of initial seroconversion. Previous studies have focused on assessing the risk of combinations of autoantibodies in cross-sectional screening designs when the actual timing of the initial seroconversion was unknown or in longitudinal studies with varied screening intervals or at/near diagnosis with T1D in those with familial risk. The relatively short interval for autoantibody screening in this study captured the sequential time of autoantibody appearance in TEDDY children from both the general population (89%) and those with a family history.

The majority of TEDDY children (77%) developed first-appearing IAA or GADA very early in life (7), as also observed in the Finnish DIPP study (8). Consistent with other studies (6,8,9), both IA-2A and ZnT8A were less likely to be a first-appearing autoantibody. Overall, ZnT8A appeared around median 3–4 years of age in the German BABYDIAB study (11) and 4–5 years of age in TEDDY, a difference that is likely due

to the predominance of FDRs in the former and a general population with increased genetic risk in the latter. The reason for this later appearance of ZnT8A is not clear; however, previous studies have reported that ZnT8A is frequently detected in those patients who progress to T1D more slowly (21,39). In TEDDY, GADA or IAA were more likely to present as a second-appearing autoantibody in children under 10 years of age. It cannot be excluded that the perceived delay in the appearance of a specific second autoantibody as it relates to the age when the first autoantibody was triggered could mark a more environmentally driven, such as a prolonged enterovirus infection (40), as compared with a genetically driven disease process.

Unlike for IAA, GADA, and IA-2A, questions remain as to how much ZnT8A contributes to risk of stage 3 T1D, particularly in those who are young with increased genetic risk. In this study, ZnT8A conferred a fivefold greater risk of progression to T1D as compared with those autoantibody-positive children who did not develop multiple autoantibodies. It is evident from our findings and other studies that ZnT8A can stratify the risk of T1D progression in familial risk (11,14,32), ethnically diverse (41), and young, high-genetic-risk populations with or without familial risk (TEDDY).

However, a large study of islet cell cytoplasmic autoantibody (ICA)-positive relatives of T1D probands did not find ZnT8A as a contributing marker of T1D in those <20 years at increased genetic risk (22). The likely reason for this inconsistency with our finding may be due to the preference of IAA, GADA, or IA-2A over ZnT8A in those with familial and genetic risk; the use of ICAs versus IAA, GADA, and IA-2A in risk determination; the high degree of concordance between GADA and ICA; and the small number ($n = 69$) of subjects with increased genetic risk <20 years of age (22).

Understanding the transition from being positive for a single autoantibody to being stage 1 (multiple autoantibodies) has both research and clinical implications. It has been shown in multiple studies that the first-appearing autoantibody is age-dependent and likely associates with different environmental triggers (5,7–9,33). Assessment of previously published environmental risk factors (34–37) shown to associate with the induction of islet autoimmunity in TEDDY were not found to be involved in further development of a second autoantibody. We, therefore, suggest that an environmental factor, such as prolonged viral shedding (40), may be sought as the etiological agent for the autoimmune reaction that is triggering the appearance of the first autoantibody and, thereby, the commencement of the autoimmune pathogenesis. The present analysis suggests that the appearance of a second autoantibody is related to other yet unknown mechanisms often referred to as epitope spreading. The present data expand our understanding of the temporal pattern by which the second autoantibody appears. Although the mechanisms need to be clarified, at least it improves prediction of progression to T1D, which advances our clinical practices for those at greatest risk and recruitment to secondary prevention clinical trials (42). Ultimately, this will result in better age-dependent autoantibody screening approaches, a reduction in symptomatic diagnoses and more strategic treatment interventions. Such specific autoantibody risk profiling will reduce heterogeneity and improve selection criteria to evaluate study objectives and interventions.

The main limitations are the select population of children at a high HLA-DR-DQ

genetic risk, the lack of extended follow-up past the age of 10 years, and the screening protocol for ZnT8A, which only measured ZnT8A in those children who developed another autoantibody (GADA, IA-2A, or IAA) further limiting our ability to determine the incidence of ZnT8A as a first-appearing autoantibody. A potential weakness of the current study is the inclusion criteria of children in four different countries with increased genetic risk for diabetes (26). The HLA-DR-DQ genotypes included 4–7.5% of newborns. However, the expected number of children reaching T1D by 18 years of age would represent <50% of the total number of children developing T1D. Certain HLA-DQ haplotypes, such as DQ5.1 and DQ6.4, are missing from the TEDDY cohort. It cannot be excluded that the pattern of IA-2A and ZnT8A as a second-appearing autoantibody may differ from the present observations.

Taken together, in children <10 years of age with genetic risk for T1D, the type of second-appearing autoantibody was associated with both genetic and familial risk, as well as varying risks of progression to T1D. All four autoantibodies under study increased the risk of stage 3 T1D. Age continues to play a critical role in the initiation of autoimmunity, development of multiple autoantibodies and progression to T1D. The importance of understanding what triggers epitope spreading and the type of autoantibody is critical in the prevention of disease progression. Further analyses including the adolescent years (>10 years) are required to better understand the transition to stage 1 (multiple autoantibodies) and how it affects the risk of stage 3 T1D, especially, in a more diverse general population with low-to-medium genetic risk.

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