Islet autoantibody type-specific titer thresholds improve stratification of risk of progression to type 1 diabetes in children

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Full Title: Islet autoantibody type-specific titer thresholds improve stratification of risk of progression to type 1 diabetes in children

Short Title: Autoantibody titers stratify diabetes risk

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Abstract

OBJECTIVE: To utilize islet autoantibody titers to improve the estimation of future type 1 diabetes risk in children.

RESEARCH DESIGN AND METHODS: Prospective cohort studies in Finland, Germany, Sweden and the US followed 24,662 children at increased genetic or familial risk to develop islet autoimmunity and diabetes. For 1,604 children with confirmed positivity, titers of autoantibodies against insulin (IAA), glutamic acid decarboxylase (GADA) and insulinoma-associated antigen-2 (IA-2A) were harmonized for diabetes risk analyses.

RESULTS: Survival analysis from time of confirmed positivity revealed markedly different 5-year diabetes risks associated with IAA (n=909), GADA (n=1076) or IA-2A (n=714), when stratified by quartiles of titer, ranging from 19% (GADA 1\textsuperscript{st} quartile) to 60% (IA-2A 4\textsuperscript{th} quartile). The minimum titer associated with a maximum difference in 5-year risk differed for each autoantibody, corresponding to the 58.6\textsuperscript{th}, 52.4\textsuperscript{th} and 10.2\textsuperscript{nd} percentile of children specifically positive for each of IAA, GADA and IA-2A, respectively. Using these autoantibody type-specific titer thresholds in the 1,481 children with all autoantibodies tested, the 5-year risk conferred by single (n=954) and multiple (n=527) autoantibodies could be stratified from 6% to 75% (p<0.0001). The thresholds effectively identified children with 50% or higher 5-year risk when considering age-specific autoantibody screening (57-65% positive predictive value and 56-74% sensitivity for ages 1-5 years). Multivariable analysis confirmed the significance of associations between the three autoantibody titers and diabetes risk, informing a childhood risk surveillance strategy.

CONCLUSIONS: This study defined islet autoantibody type-specific titer thresholds that significantly improved type 1 diabetes risk stratification in children.
Introduction

The development of islet autoantibodies is known to precede onset of clinical type 1 diabetes (diabetes). However, the time interval from initial seroconversion to the onset of clinical symptoms varies among individuals from weeks to many years, creating uncertainty in how to monitor for metabolic instability as well as when to potentially intervene with immunotherapy. The age at seroconversion and the number and combination of specific islet autoantibodies present are known to be associated with the duration of progression from seroconversion to onset of diabetes [1]. However, the association between islet autoantibody titers and progression to diabetes is less well understood, and the results of prior studies are partly inconsistent. One of the earliest such studies showed an association between higher peak titer of islet cell antibodies (ICA) and higher risk of developing diabetes [2]. In more recent studies, associations between higher titers of autoantibodies to insulin (IAA) and insulinoma-associated antigen-2 (IA-2A) have been observed to be associated with faster progression to diabetes [3–8]. However, one study found these associations for IAA but not for IA-2A [9], and another reported that lower initial IAA titers predicted slower progression to diabetes [10]. Yet another recent study estimated that the association between autoantibody titers and progression to diabetes was time-constant for IA-2A but decreased over time for IAA [11]. For autoantibody titers of glutamic acid decarboxylase (GADA), the results have also been mixed. Some studies have found no significant association between the risk of developing diabetes and GADA titers [3,8,9]. Notably, one study reported that higher initial GADA titers were associated with more rapid progression to diabetes [6]; another found an association between GADA titers and progression to diabetes that decreased over time [11]. Despite the varying results, all evidence seems to suggest that antibody titers may be informative for stratification of diabetes risk in islet autoantibody-positive
individuals. Although other autoantibody characteristics, such as immunoglobulin IgG subclass, epitope specificity, and binding affinity may also be useful in stratifying diabetes risk, they require additional testing [12]. Autoantibody titer is a simple marker and only needs one quantitative assay. However, titer measurements are not standardized between most of the currently used islet autoantibody assays; harmonization of quantitative results is thus required. In this study, we assessed and quantified the association between the titers of different islet autoantibodies (IAA, GADA, IA-2A) and the risk of progression to diabetes, using harmonized titer values from our large prospective T1DI (Type 1 Diabetes Intelligence) study cohort [13]. We focused our analysis on the time point of seroconversion, more specifically the time at which newly detected islet autoantibody positivity was confirmed in a second consecutive sample. Our goal was to leverage islet autoantibody titers to refine diabetes risk stratification for children who developed confirmed-positive islet autoantibodies.

**Research Design and Methods**

**Study Population**

Prospective cohorts in Finland (DIPP [14]), Germany (BABYDIAB [15]), Sweden (DiPiS [16]), and the United States (DAISY [17], DEW-IT [18]) have followed 24,662 children at increased genetic and familial risk for development of islet autoantibodies and diabetes, from close to birth for a period of 15 years, or until their diagnosis. Data from these studies were combined and harmonized in the T1DI study cohort [13]. Only those children with confirmed positivity to IAA, GADA, or IA-2A and with autoantibody titer measurements before diagnosis of diabetes, or the end of study follow-up period, were selected for analysis (Supplemental Figure S1). This cohort (“Study Cohort 1”) had 1,604 children, of whom 600 (37.4%) developed diabetes (Supplemental
Table S1). There was a total of 32,660 visits with a mean (standard deviation) of the time interval between successive visits of 0.53 (0.71) years. A more constrained second cohort, consisting of children with complete autoantibody titer measurements for all three autoantibodies, in the first overall islet autoantibody-positive and in the second consecutive positive serum sample, were selected for additional analysis (Supplemental Figure S1). This second cohort (“Study Cohort 2”) had 1,481 children, of whom 570 (38.5%) developed diabetes (Supplemental Table S2). All T1DI constituent studies were approved by their respective ethics review boards.

**Islet Autoantibody Measurements**

The methods used by each study to measure IAA, GADA, and IA-2A have been previously described, and are summarized in the supplement of [13]. Each of the studies and their laboratories have participated - with satisfactory results - in both the Diabetes Autoantibody Standardization program (DASP [19–21]) and the Islet Autoantibody Standardization program (IASP [22]) proficiency workshops. Because in the T1DI study cohort, titer values for the same autoantibody may originate from different assays with different units, they are not directly comparable. Therefore, as described in Supplemental Section S1 and Supplemental Figure S2, all autoantibody titer measurements for IAA, GADA, and IA-2A were converted to multiples of upper limit of normal (mULN) to facilitate comparisons and combined for analysis. Autoantibodies to zinc transporter 8 (ZnT8A) were not consistently measured across all constituent T1DI studies. Several of the studies only measured ZnT8A if the child tested positive for one or more of the other three autoantibodies or had developed diabetes; as a result, ZnT8A was not included in our analyses.
Confirmed autoantibody positivity was defined as a positive test result (for the same autoantibody type) in at least two consecutive samples, regardless of the time interval between the visits. The first and second of these two consecutive visits will be hereby referred to as the initial visit and confirmatory visit, respectively (Supplemental Figure S3). The mean time intervals (standard deviation), in years, between the initial and confirmatory visits were 0.4 (0.5), 0.5 (0.5), and 0.4 (0.7) for IAA, GADA, and IA-2A respectively. The mean age (standard deviation), in years, at the confirmatory visit were 5.4 (4.1), 6.1 (4.1), and 5.7 (3.9) for positivity to IAA, GADA, and IA-2A respectively (Supplemental Table S1).

In our analyses, we focused on the autoantibody data from the confirmatory visit (when autoantibody positivity was first confirmed) rather than the initial visit (when autoantibody positivity was first detected) because we assumed that the autoantibody response would be more robust and mature in confirmatory testing and would better reflect the situation in practice in a screening scenario.

**Outcome Definition**

Type 1 diabetes diagnosis was based on the World Health Organization and American Diabetes Association criteria [23]. The main outcome of interest is the time-to-diabetes from the time of the confirmatory-visit-for-positivity to IAA, GADA, or IA-2A. Specifically, the outcome is defined as the time, in years, from the confirmatory visit for one of the three specific autoantibodies, to the time of diagnosis of diabetes for events, or the time of last follow-up (censoring time) for non-events.

**Statistical Analyses**

Five different analyses were performed, each focused on addressing a different question.
1. How well can islet autoantibody titers stratify diabetes risk?

Children in Study Cohort 1 were stratified based on autoantibody-specific titer quartiles from their confirmatory visit for autoantibody positivity. Time-to-event analysis was then used to examine whether progression from the confirmatory visit to clinical diabetes was associated with the autoantibody titer. Kaplan-Meier (KM) estimates with 95% confidence intervals were used to estimate diabetes risk from the confirmatory visit; log-rank tests were used to establish statistical differences between the strata in the KM analysis.

2. What islet autoantibody type-specific titer threshold maximizes 5-year diabetes risk stratification?

Study Cohort 1 was used to identify the lowest autoantibody type-specific titer threshold at the confirmatory visit that maximized the difference in 5-year diabetes risk between two groups, i.e., those with titers at-or-above the threshold vs. those with titers below the threshold. To do this, all possible threshold values between 1.0 and T (where T is the titer value corresponding to the 75th percentile of the respective autoantibody-positive cohort) were considered separately for IAA, GADA, and IA-2A. For each threshold value, the cohort was partitioned into two groups, as described above, and KM analysis was performed to estimate diabetes risk for both groups from the confirmatory visit. Next, the 5-year diabetes risk for each group was extracted, and the difference between them computed. Finally, the lowest titer threshold value resulting in the maximum risk difference was selected.

3. How well can the autoantibody type-specific titer thresholds stratify diabetes risk for single and multiple islet autoantibody-positive children?

The autoantibody type-specific titer thresholds were then used in a KM survival analysis
using Study Cohort 2, to stratify diabetes risk for children with single and multiple islet autoantibody-positive status. Information from the earliest confirmatory visit in the child’s history was used to determine the single and multiple islet autoantibody status. Single autoantibody-positive children were stratified into two groups: those with titers at-or-above the autoantibody type-specific threshold and those with titers below the threshold. Multiple autoantibody-positive children were stratified into four groups: those with zero, one, two, or three titers at-or-above the autoantibody type-specific thresholds. Log-rank tests were used to establish statistical differences between the strata.

4. **How significant are the associations between autoantibody titers and diabetes?**

Next, a series of multivariable analyses were performed to quantify the significance of autoantibody titers from the earliest confirmed autoantibody positivity, using Study Cohort 2. Hazard ratios (HRs) - and corresponding 95% confidence intervals of association - between titers and diabetes were estimated, using Cox proportional hazards regression. An initial model analyzed the association between autoantibody positivity (present or absent) at the earliest confirmatory visit and diabetes risk. The model was adjusted for human leukocyte antigen (HLA) risk group, sex, and age at the earliest confirmatory visit, and stratified by study site. As described in the supplement of [13], genotypes from individual studies were harmonized into four HLA risk groups (A, B, C, D), ordered by decreasing risk. Autoantibody positivity indicators for IAA, GADA, IA-2A, at the earliest confirmatory visit, were the primary predictors. A second model analyzed the association between autoantibody titers and diabetes risk. In this model, the log transformed autoantibody titers (log mULN) for IAA, GADA, IA-2A, at the earliest confirmatory visit, were added to the initial model as the primary predictors. The proportional hazards assumption was tested using the
Schoenfeld test [24]. For covariates that did not satisfy the proportional hazards assumption at the 0.05 significance level, time-varying coefficients were used with time modeled linearly [25,26]. P-values corrected for multiple comparisons using the Benjamini-Hochberg method were reported [27].

5. **How well can the autoantibody type-specific titer thresholds identify children with high diabetes risk based on autoantibody screening at different ages?**

Finally, an application of the autoantibody type-specific titer thresholds to identify children at high risk of developing diabetes in the next five years was explored, for potential clinical trial recruitment. Specifically, the ability to use these titer thresholds to stratify diabetes risk (based on results from autoantibody screening at different age ranges), was assessed. The following age ranges were explored: 1-2.0, 2-3.0, 3-4.0, 4-5.0, 5-10.0, and 10+ years. For each age range, children in Study Cohort 1 with at least one autoantibody titer measurement, and without already being diagnosed with diabetes, were included for analysis. These children were stratified based only on the observed autoantibody titers in that age range (when multiple measurements of the same autoantibody were available for a child within the age range, the earliest one was used). Each child was placed into one of twelve possible strata defined by single or multiple autoantibody positivity and the combination of IAA, GADA, and IA-2A titers at-or-above the autoantibody type-specific threshold. KM survival analysis was then performed to estimate the risk of diabetes from the time of the autoantibody measurement, for each age range and each stratum. Children belonging to strata that have an estimated 5-year diabetes risk \( \geq 50\% \) were labeled as “high-risk”. Prediction performance was measured using inverse probability of censoring weighted
(IPCW [28,29]) positive predictive value (PPV), negative predictive value (NPV), sensitivity, and specificity.

Analyses were performed using Python (scikit-learn, scikit-survival) and R (survival, survminer) software [30,31].

Results

Stratification of diabetes risk by islet autoantibody titer quartiles

Survival analysis from time of confirmed positivity revealed markedly different 5-year diabetes risks associated with IAA (n=909), GADA (n=1076) or IA-2A (n=714), when stratified by quartiles of titer, ranging from 19% (GADA 1st quartile) to 60% (IA-2A 4th quartile) (Supplemental Figure S4). Histogram distributions and quartile thresholds of autoantibody titers, at the confirmatory visit for positivity to IAA, GADA, and IA-2A, are shown in Supplemental Figure S5.

Determination of autoantibody type-specific titer thresholds that maximize 5-year diabetes risk discrimination

The results of this analysis for IAA, GADA, and IA-2A are shown in Figure 1 A, B, and C, respectively. The minimum titer value associated with a maximum difference in 5-year diabetes risk differed for each autoantibody type: \( T_{\text{IAA}} = 3.6 \text{ mULN} \), \( T_{\text{GADA}} = 5.4 \text{ mULN} \), and \( T_{\text{IA-2A}} = 2.5 \text{ mULN} \). These titer threshold levels corresponded to 58.6th, 52.4th, and 10.2nd percentile of children positive for IAA, GADA, and IA-2A, respectively.

Using these thresholds, children positive to each autoantibody type were stratified into two groups: those with titers at-or-above threshold and those with titers below threshold. This stratification resulted in significantly different 5-year diabetes risks for all three autoantibody types \((\text{all } p<0.0001)\) (Supplemental Figure S6).
Improved stratification of diabetes risk by autoantibody type-specific titer thresholds

Stratifying single and multiple islet autoantibody-positive children (determined at time of the earliest confirmatory visit) using the autoantibody type-specific thresholds resulted in significantly different 5-year diabetes risks. For single autoantibody-positive children (n=954), those with antibody titers at-or-above the autoantibody type-specific threshold had a 5-year diabetes risk of 21.9% [95% CI, 17.0-26.4%] (n=364), compared to 6.1% [3.9-8.3%] (n=590) for those with titers below the threshold (Figure 2A). For multiple autoantibody-positive children (n=527), those with zero (n=49), one (n=202), two (n=216), and three (n=60) antibody titers at-or-above the autoantibody type-specific thresholds had a 5-year diabetes risk of 24.7% [95% CI, 10.1-36.9%], 41.2% [33.7-47.9%], 55.7% [48.2-62.1%], and 75.1% [61.0-84.1%], respectively (Figure 2B). The corresponding follow-up times from the earliest confirmatory visit to 50% cumulative progression to diabetes were 8.5yr [95% CI, 7.1-15.0yr], 5.8yr [5.3-6.8yr], 4.0yr [3.3-5.1yr], and 2.3yr [1.6-3.3yr], respectively. A total of 276 children had a 50% or greater risk of developing diabetes within 4 years of first confirmed autoantibody positivity.

Association between islet autoantibody titer and diabetes risk in multivariable analysis

The multivariable regression models that analyzed the association between autoantibodies at the earliest confirmatory visit and diabetes risk are shown in Supplemental Table S3. Model 1 used the autoantibody positivity indicators as predictors. Time-dependent covariates were used for both GADA and IA-2A positivity since they did not satisfy the proportional hazards assumption. At the earliest confirmatory visit, the adjusted HR for GADA positivity was significant (HR 1.43; 95% CI 1.05-1.95; p=0.02) and increased over time (HR 1.06 per year; 95% CI 1.01-1.12; p=0.02). The adjusted HR for IA-2A positivity was significant (HR 3.93; 95% CI 2.95-5.23; p<0.0001) but decreased over time (HR 0.95 per year; 95% CI 0.90-0.99; p=0.02). The adjusted
HR for IAA positivity was also significant (HR 2.10; 95% CI 1.74-2.55; p<0.0001). Age at the earliest confirmatory visit and HLA risk group were also significant. Model 2 adds the corresponding autoantibody titers as predictors. Note that all three of the autoantibody positivity indicators were no longer significant once the autoantibody titers were added. Time dependent covariates were used for IAA titer, since it was the only significant covariate that did not satisfy the proportional hazards assumption. At the earliest confirmatory visit, the adjusted HR for IAA titer was significant (HR 1.37; 95% CI 1.24-1.51; p<0.001) and decreased over time (HR 0.98 per year; 95% CI 0.97-1.0; p =0.01). The adjusted HR for GADA titer (HR1.18; 95% CI 1.11-1.25; p<0.001) and IA-2A titer (HR1.17; 95% CI 1.10-1.24; p< 0.001) were also significant. Age at the earliest confirmatory visit and HLA risk group remained significant. Finally, Model 2 showed higher concordance (standard error) than Model 1: 0.78 (0.01) versus 0.75 (0.01).

**Effectiveness of identifying islet autoantibody-positive children at high risk for diabetes at different ages by using autoantibody type-specific titer thresholds**

The twelve strata, resulting from all possible groupings of single or multiple autoantibody positivity and the combinations of IAA, GADA, IA-2A titers above threshold, and their estimated 5-year diabetes risks are shown in Figure 3 for each age range (the underlying KM analyses are shown in Supplemental Figure S7). Strata that have a 5-year diabetes risk ≥ 50% are considered “high-risk” and are shaded in red. Supplemental Table S4 lists, for each age range, the individual high-risk strata, the “composite high-risk criteria” defined by forming a union of the individual high-risk strata, the total number of children, the number that progressed to diabetes within 5 years, the number of high-risk children identified using the composite high-risk criteria, and the associated PPV, NPV, sensitivity, and specificity performance metrics. There were 167, 289, 231, 283, 60, and 35 high-risk children identified for the age ranges 1-2.0, 2-3.0,
3-4.0, 4-5.0, 5-10.0, and 10+ years, respectively. The PPV was consistent across the age groups ranging from 55% to 65%. Sensitivity ranged from 56% and 74% between ages 1-5 years but dropped significantly to 12% and 14% for ages 5-10 and 10+ years, respectively. As the age of the child being screened increased, not only were more stringent autoantibody criteria needed to identify those with high diabetes risk, but it also became more difficult to reliably identify them. A summary of the process to identify high diabetes risk children is illustrated in the decision flowchart in Figure 4.

Conclusions

This study showed that islet autoantibody titers can stratify risk of progression to diabetes in children, beyond information about the number and type of islet autoantibodies present. Furthermore, these titers matter in different ways for different autoantibodies, and customized islet autoantibody type-specific titer thresholds could be defined that maximized discrimination of the 5-year diabetes risk. The combination of these titer thresholds effectively identified among islet autoantibody-positive children those with a 50% or higher 5-year risk of diabetes who could be potential candidates for participation in intervention trials. The study used data from a large cohort of children prospectively followed in five different birth cohorts, harmonized autoantibody titers across these five studies, and combined them for analysis. Stratification of diabetes risk based on islet autoantibody titer quartiles showed for each of IAA, GADA and IA-2A that higher titers were associated with higher diabetes risk, complementing findings from prior studies [1,3–8]. Islet autoantibodies with high titers usually involve multiple IgG subclasses and are directed against multiple epitopes on the target antigen, likely reflecting a more intense and prolonged autoimmune response and associated with the
progression of diabetes development [12]. The current analysis also revealed that different autoantibody types exhibited different patterns (Supplemental Figure S4). Based on the 5-year diabetes risk, there was no significant separation between neighboring quartiles for IAA, indicating a relatively smooth risk distribution as a function of titer. For GADA, the only significant separation was between the second and third quartiles, indicating a bimodal risk distribution with a gap around the median. For IA-2A, there was only separation between the first and second quartiles, indicating a bimodal risk distribution with a gap around the first quartile. Plots of the cohort percentile as a function of titer threshold at confirmed positivity (Figure 1, top panel) also revealed different distributional behaviors. For IAA, the concave-shaped plot indicated that there were more IAA-positive children with lower IAA titers. The linear-shaped GADA plot indicated an even GADA titer distribution. For IA-2A, the convex-shaped plot indicated that IA-2A-positive children tended to have higher IA-2A titers.

In order to identify islet autoantibody type-specific titer thresholds we used a novel analytical approach that has potential advantages. Specifically, our method was a data-driven approach to automatically identify thresholds that maximize a given outcome. It does this by scanning over the possible threshold values, e.g., increasing autoantibody titers, splitting the cohort based on each threshold value, performing survival analyses on the two resulting groups, and computing the outcome. Using difference in 5-year risk of diabetes as an illustrative outcome of interest, the islet autoantibody type-specific titer thresholds were identified. Furthermore, translation of the islet autoantibody type-specific titer thresholds into percentiles of autoantibody-positive children is important because it allows the thresholds to be applied to external datasets that may have different assay characteristics and normalization methods. The appropriate threshold will depend on the application; notably, the method developed to identify
the thresholds is flexible and generalizable: it can be easily reconfigured to accommodate different outcomes. It may even be adapted for use with other quantified biomarkers such as plasma glucose or glycated hemoglobin. To demonstrate this, we selected a different outcome (e.g., difference in 3-year diabetes risk) and re-ran the analysis. A different set of autoantibody type-specific thresholds were identified that maximized the stratification of 3-year diabetes risk (Supplemental Figure S8) and selected smaller groups of children with higher risk of fast progression to diabetes (Supplemental Figures S9 and S10). It should be noted, however, that because the thresholds were determined based on our study cohort, there may be some uncertainty when extrapolating to other data sets and they may not be as predictive when applied to other cohorts.

The multivariable regression analysis found that the autoantibody positivity indicators for IAA, GADA, IA-2A, at the earliest confirmatory visit, were all significantly associated with diabetes risk. However, when the corresponding autoantibody titers were added, these indicator variables were no longer significant. Instead, all three of the titer variables became significant, indicating that the titers contain more information than the indicators. The HR was time-constant for GADA and IA-2A but decreased over time for IAA. Prior work has found associations between titers and progression to diabetes that were time-constant for IA-2A, but decreased over time for GADA and IAA [11].

The age-based autoantibody screening simulation analysis was able to identify children with a high risk of developing diabetes, using autoantibody positivity and the islet autoantibody type-specific titer thresholds. Of note, the presence of IA-2A above titer threshold alone was sufficient to identify high diabetes risk in children aged 2-5 years, even in the absence of IAA or GADA. It
is known that IA-2A usually occurs together with autoantibodies against other beta cell antigens and is therefore highly specific and predictive for progression to clinical diabetes [3,32].

Overall, the results of this study may contribute to improved risk counselling for families of affected children and improved screening for participants for intervention therapy trials aimed at preventing or delaying progression to clinical disease. Since titers add value beyond autoantibody type and number, islet autoantibody standardization programs (e.g., IASP) should continue to focus on improving titer standardization, to facilitate quantitative comparisons across assays and study sites.

This study has some limitations. First, the autoantibody titers were measured using different assays across the study sites. Although the titers were harmonized, some residual biases may remain. In addition, the current data are based on radio binding assay results. Islet autoantibody type-specific titer thresholds and respective percentiles of positives may need to be adjusted for other assay formats such as those based on electrochemiluminescence [33], luciferase immunoprecipitation system [34], or agglutination-PCR [35] technology. Second, due to differences in the visit intervals of the study protocols, it is possible that the actual time of the earliest autoantibody positivity was missed, with the consequence that the measured time is biased. Off-schedule visits may also impact the timing of the confirmatory visit. Third, only children with increased genetic and familial risk for development of islet autoimmunity and diabetes were enrolled into the studies, and the study populations were predominantly Caucasian, which may limit generalizability of the results. Fourth, the analyses have not been validated on an independent cohort.

There are several possible directions for future work. First, the analyses should be replicated in higher time resolution datasets with more frequent prospective follow-up (e.g., TEDDY [36]).
Second, validation in independent cohorts with broader population inclusion criteria (e.g., Fr1da [37] or ASK studies [38]) should be undertaken. Third, the age-based risk stratification performance should be validated in a cross-sectional study. Fourth, the utility of islet autoantibody titers as a continuous variable should be further explored in diabetes risk prediction [39] and disease progression modeling [40].

In summary, this study harmonized islet autoantibody titers across multiple birth cohorts, combined them for analysis, and defined autoantibody type-specific titer thresholds that significantly improved type 1 diabetes risk stratification in children.
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**Author contributions:** KN, PA, WH conceived and designed the study. KN, HS, VA, RV, JT, MM, KW, WH, PA acquired, analyzed, and interpreted the data. KN, PA drafted the manuscript. KN, HS, VA, RV, JT, MM, ML, KW, BF, FM, WH, PA critically revised the manuscript for important intellectual content. All authors gave final approval of the version to be submitted.
References


Figure Captions

**Figure 1**: Identifying autoantibody type-specific titer thresholds for IAA (A), GADA (B), and IA-2A (C). Top panel: The size of the red cohort (titer $\geq$ threshold) and the green cohort (titer $<$ threshold) for each autoantibody titer threshold level. Middle panel: 5-year risk of diabetes and 95% confidence intervals from the time of the confirmatory visit for autoantibody positivity for the red and green cohorts for each titer threshold level. Bottom panel: The difference in the 5-year diabetes risk between the red and green cohorts for each titer threshold level. An arrow marks the lowest titer threshold level where there is a maximum risk difference between the cohorts and the threshold covers up to 75% of the cohort ($T_{IAA} = 3.6$ mULN, $T_{GADA} = 5.4$ mULN, and $T_{IA-2A} = 2.5$ mULN). The percentile of children who tested positive for the respective autoantibody corresponding to the final titer threshold is highlighted in the top panel ($T_{IAA} \rightarrow 58.6\%$, $T_{GADA} \rightarrow 52.4\%$, and $T_{IA-2A} \rightarrow 10.2\%$). IAA indicates insulin autoantibodies; GADA, glutamic acid decarboxylase autoantibodies; IA-2A, insulinoma-associated antigen-2 autoantibodies; mULN, multiples of upper limit of normal; DM, diabetes mellitus.

**Figure 2**: Progression to diabetes from the time of the earliest confirmatory visit in children with single and multiple autoantibody positivity. Stratification is based on the autoantibody titer measured at the earliest confirmatory visit and the identified autoantibody type-specific titer thresholds ($T_{IAA} = 3.6$ mULN, $T_{GADA} = 5.4$ mULN, $T_{IA-2A} = 2.5$ mULN). (A) Single autoantibody-positive children are partitioned into two groups: those with autoantibody titer below threshold ($t < T$) and those with titer at-or-above threshold ($t \geq T$). (B) Multiple autoantibody-positive children are partitioned into four mutually exclusive groups: those with no autoantibody titer at-or-above threshold ($0IAb \geq T$), those with one autoantibody titer at-or-above threshold ($1IAb \geq T$), those with two autoantibody titers at-or-above threshold ($2IAb \geq T$), and those with all three autoantibody titers at-or-above threshold ($3IAb \geq T$). The dashed vertical line marks the 5-year follow-up time point. IAA indicates insulin autoantibodies; GADA, glutamic acid decarboxylase autoantibodies; IA-2A, insulinoma-associated antigen-2 autoantibodies; mULN, multiples of upper limit of normal.
Figure 3: The 5-year risk of type 1 diabetes and 95% confidence intervals in subjects who developed confirmed-positive islet autoantibodies, stratified by single or multiple autoantibody positivity and the combination of IAA, GADA, IA-2A titers above thresholds \(T_{\text{IAA}} = 3.6\) mULN, \(T_{\text{GADA}} = 5.4\) mULN \(T_{\text{IA-2A}} = 2.5\) mULN) for screening at different age ranges (A: 1-2.0 y, B: 2-3.0 y, C: 3-4.0 y, D: 4-5.0 y, E: 5-10.0 y, F: 10+ y). The 12 strata are:

- **S:0T:--** = single positive, no autoantibodies above titer threshold
- **S:1T:GADA** = single positive, one (GADA) above titer threshold
- **S:1T:IAA** = single positive, one (IAA) above titer threshold
- **S:1T:IA-2A** = single positive, one (IA-2A) above titer threshold
- **M:0T:--** = multiple positive, no autoantibodies above titer threshold
- **M:1T:GADA** = multiple positive, one (GADA) above titer threshold
- **M:1T:IAA** = multiple positive, one (IAA) above titer threshold
- **M:1T:IA-2A** = multiple positive, one (IA-2A) above titer threshold
- **M:2T:GADA,IAA** = multiple positive, two (GADA, IAA) above titer threshold
- **M:2T:GADA,IA-2A** = multiple positive, two (GADA, IA-2A) above titer threshold
- **M:2T:IA-2A,IAA** = multiple positive, two (IA-2A, IAA) above titer threshold
- **M:3T:GADA,IA-2A,IAA** = multiple positive, all three above titer threshold

The number of subjects in each stratum is shown at the base of each bar. The dashed vertical red lines mark the 50% 5-year risk of diabetes level. Strata that exceed that risk level are classified as “high-risk” and shaded red. IAA indicates insulin autoantibodies; GADA, glutamic acid decarboxylase autoantibodies; IA-2A, insulinoma-associated antigen-2 autoantibodies; mULN, multiples of upper limit of normal.

Figure 4: A proposed flowchart to discover islet autoantibody-positive children and then evaluate their antibody titer to identify those at high risk (≥50%) of developing type 1 diabetes within 5 years. A child can enter the flowchart by autoantibody testing at any age via a blue arrow and appropriate blue box. Those with antibodies fulfilling the titer criteria shown in the corresponding grey box are at high risk and could be considered for intervention therapy trials or close glycemic monitoring. Islet autoantibodies tested include insulin autoantibodies (IAA), glutamic acid decarboxylase autoantibodies (GADA), and insulinoma-associated antigen-2 autoantibodies (IA-2A).
Full Title: Islet autoantibody type-specific titer thresholds improve stratification of risk of progression to type 1 diabetes in children

Short Title: Autoantibody titers stratify diabetes risk

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Abstract

OBJECTIVE: To utilize islet autoantibody titers to improve the estimation of future type 1 diabetes risk in children.

RESEARCH DESIGN AND METHODS: Prospective cohort studies in Finland, Germany, Sweden and the US followed 24,662 children at increased genetic or familial risk to develop islet autoimmunity and diabetes. For 1,604 children with confirmed positivity, titers of autoantibodies against insulin (IAA), glutamic acid decarboxylase (GADA) and insulinoma-associated antigen-2 (IA-2A) were harmonized for diabetes risk analyses.

RESULTS: Survival analysis from time of confirmed positivity revealed markedly different 5-year diabetes risks associated with IAA (n=909), GADA (n=1076) or IA-2A (n=714), when stratified by quartiles of titer, ranging from 19% (GADA 1st quartile) to 60% (IA-2A 4th quartile). The minimum titer associated with a maximum difference in 5-year risk differed for each autoantibody, corresponding to the 58.6th, 52.4th and 10.2nd percentile of children specifically positive for each of IAA, GADA and IA-2A, respectively. Using these autoantibody type-specific titer thresholds in the 1,481 children with all autoantibodies tested, the 5-year risk conferred by single (n=954) and multiple (n=527) autoantibodies could be stratified from 6% to 75% (p<0.0001). The thresholds effectively identified children with 50% or higher 5-year risk when considering age-specific autoantibody screening (57-65% positive predictive value and 56-74% sensitivity for ages 1-5 years). Multivariable analysis confirmed the significance of associations between the three autoantibody titers and diabetes risk, informing a childhood risk surveillance strategy.

CONCLUSIONS: This study defined islet autoantibody type-specific titer thresholds that significantly improved type 1 diabetes risk stratification in children.
Introduction

The development of islet autoantibodies is known to precede onset of clinical type 1 diabetes (diabetes). However, the time interval from initial seroconversion to the onset of clinical symptoms varies among individuals from weeks to many years, creating uncertainty in how to monitor for metabolic instability as well as when to potentially intervene with immunotherapy. The age at seroconversion and the number and combination of specific islet autoantibodies present are known to be associated with the duration of progression from seroconversion to onset of diabetes [1]. However, the association between islet autoantibody titers and progression to diabetes is less well understood, and the results of prior studies are partly inconsistent. One of the earliest such studies showed an association between higher peak titer of islet cell antibodies (ICA) and higher risk of developing diabetes [2]. In more recent studies, associations between higher titers of autoantibodies to insulin (IAA) and insulinoma-associated antigen-2 (IA-2A) have been observed to be associated with faster progression to diabetes [3–8]. However, one study found these associations for IAA but not for IA-2A [9], and another reported that lower initial IAA titers predicted slower progression to diabetes [10]. Yet another recent study estimated that the association between autoantibody titers and progression to diabetes was time-constant for IA-2A but decreased over time for IAA [11]. For autoantibody titers of glutamic acid decarboxylase (GADA), the results have also been mixed. Some studies have found no significant association between the risk of developing diabetes and GADA titers [3,8,9]. Notably, one study reported that higher initial GADA titers were associated with more rapid progression to diabetes [6]; another found an association between GADA titers and progression to diabetes that decreased over time [11]. Despite the varying results, all evidence seems to suggest that antibody titers may be informative for stratification of diabetes risk in islet autoantibody-positive
individuals. Although other autoantibody characteristics, such as immunoglobulin IgG subclass, epitope specificity, and binding affinity may also be useful in stratifying diabetes risk, they require additional testing [12]. Autoantibody titer is a simple marker and only needs one quantitative assay. However, titer measurements are not standardized between most of the currently used islet autoantibody assays; harmonization of quantitative results is thus required.

In this study, we assessed and quantified the association between the titers of different islet autoantibodies (IAA, GADA, IA-2A) and the risk of progression to diabetes, using harmonized titer values from our large prospective T1DI (Type 1 Diabetes Intelligence) study cohort [13]. We focused our analysis on the time point of seroconversion, more specifically the time at which newly detected islet autoantibody positivity was confirmed in a second consecutive sample. Our goal was to leverage islet autoantibody titers to refine diabetes risk stratification for children who developed confirmed-positive islet autoantibodies.

**Research Design and Methods**

**Study Population**

Prospective cohorts in Finland (DIPP [14]), Germany (BABYDIAB [15]), Sweden (DiPiS [16]), and the United States (DAISY [17], DEW-IT [18]) have followed 24,662 children at increased genetic and familial risk for development of islet autoantibodies and diabetes, from close to birth for a period of 15 years, or until their diagnosis. Data from these studies were combined and harmonized in the T1DI study cohort [13]. Only those children with confirmed positivity to IAA, GADA, or IA-2A and with autoantibody titer measurements before diagnosis of diabetes, or the end of study follow-up period, were selected for analysis (Supplemental Figure S1). This cohort (“Study Cohort 1”) had 1,604 children, of whom 600 (37.4%) developed diabetes (Supplemental
There was a total of 32,660 visits with a mean (standard deviation) of the time interval between successive visits of 0.53 (0.71) years. A more constrained second cohort, consisting of children with complete autoantibody titer measurements for all three autoantibodies, in the first overall islet autoantibody-positive and in the second consecutive positive serum sample, were selected for additional analysis (Supplemental Figure S1). This second cohort (“Study Cohort 2”) had 1,481 children, of whom 570 (38.5%) developed diabetes (Supplemental Table S2). All T1DI constituent studies were approved by their respective ethics review boards.

**Islet Autoantibody Measurements**

The methods used by each study to measure IAA, GADA, and IA-2A have been previously described, and are summarized in the supplement of [13]. Each of the studies and their laboratories have participated - with satisfactory results - in both the Diabetes Autoantibody Standardization program (DASP [19–21]) and the Islet Autoantibody Standardization program (IASP [22]) proficiency workshops. Because in the T1DI study cohort, titer values for the same autoantibody may originate from different assays with different units, they are not directly comparable. Therefore, as described in Supplemental Section S1 and Supplemental Figure S2, all autoantibody titer measurements for IAA, GADA, and IA-2A were converted to multiples of upper limit of normal (mULN) to facilitate comparisons and combined for analysis.

Autoantibodies to zinc transporter 8 (ZnT8A) were not consistently measured across all constituent T1DI studies. Several of the studies only measured ZnT8A if the child tested positive for one or more of the other three autoantibodies or had developed diabetes; as a result, ZnT8A was not included in our analyses.
Confirmed autoantibody positivity was defined as a positive test result (for the same autoantibody type) in at least two consecutive samples, regardless of the time interval between the visits. The first and second of these two consecutive visits will be hereby referred to as the initial visit and confirmatory visit, respectively (Supplemental Figure S3). The mean time intervals (standard deviation), in years, between the initial and confirmatory visits were 0.4 (0.5), 0.5 (0.5), and 0.4 (0.7) for IAA, GADA, and IA-2A respectively. The mean age (standard deviation), in years, at the confirmatory visit were 5.4 (4.1), 6.1 (4.1), and 5.7 (3.9) for positivity to IAA, GADA, and IA-2A respectively (Supplemental Table S1).

In our analyses, we focused on the autoantibody data from the confirmatory visit (when autoantibody positivity was first confirmed) rather than the initial visit (when autoantibody positivity was first detected) because we assumed that the autoantibody response would be more robust and mature in confirmatory testing and would better reflect the situation in practice in a screening scenario.

Outcome Definition

Type 1 diabetes diagnosis was based on the World Health Organization and American Diabetes Association criteria [23]. The main outcome of interest is the time-to-diabetes from the time of the confirmatory-visit-for-positivity to IAA, GADA, or IA-2A. Specifically, the outcome is defined as the time, in years, from the confirmatory visit for one of the three specific autoantibodies, to the time of diagnosis of diabetes for events, or the time of last follow-up (censoring time) for non-events.

Statistical Analyses

Five different analyses were performed, each focused on addressing a different question.
1. **How well can islet autoantibody titers stratify diabetes risk?**

Children in Study Cohort 1 were stratified based on autoantibody-specific titer quartiles from their confirmatory visit for autoantibody positivity. Time-to-event analysis was then used to examine whether progression from the confirmatory visit to clinical diabetes was associated with the autoantibody titer. Kaplan-Meier (KM) estimates with 95% confidence intervals were used to estimate diabetes risk from the confirmatory visit; log-rank tests were used to establish statistical differences between the strata in the KM analysis.

2. **What islet autoantibody type-specific titer threshold maximizes 5-year diabetes risk stratification?**

Study Cohort 1 was used to identify the lowest autoantibody type-specific titer threshold at the confirmatory visit that maximized the difference in 5-year diabetes risk between two groups, i.e., those with titers at-or-above the threshold vs. those with titers below the threshold. To do this, all possible threshold values between 1.0 and T (where T is the titer value corresponding to the 75th percentile of the respective autoantibody-positive cohort) were considered separately for IAA, GADA, and IA-2A. For each threshold value, the cohort was partitioned into two groups, as described above, and KM analysis was performed to estimate diabetes risk for both groups from the confirmatory visit. Next, the 5-year diabetes risk for each group was extracted, and the difference between them computed. Finally, the lowest titer threshold value resulting in the maximum risk difference was selected.

3. **How well can the autoantibody type-specific titer thresholds stratify diabetes risk for single and multiple islet autoantibody-positive children?**

The autoantibody type-specific titer thresholds were then used in a KM survival analysis
using Study Cohort 2, to stratify diabetes risk for children with single and multiple islet autoantibody-positive status. Information from the earliest confirmatory visit in the child’s history was used to determine the single and multiple islet autoantibody status. Single autoantibody-positive children were stratified into two groups: those with titers at-or-above the autoantibody type-specific threshold and those with titers below the threshold. Multiple autoantibody-positive children were stratified into four groups: those with zero, one, two, or three titers at-or-above the autoantibody type-specific thresholds. Log-rank tests were used to establish statistical differences between the strata.

4. **How significant are the associations between autoantibody titers and diabetes?**

Next, a series of multivariable analyses were performed to quantify the significance of autoantibody titers from the earliest confirmed autoantibody positivity, using Study Cohort 2. Hazard ratios (HRs) - and corresponding 95% confidence intervals of association - between titers and diabetes were estimated, using Cox proportional hazards regression. An initial model analyzed the association between autoantibody positivity (present or absent) at the earliest confirmatory visit and diabetes risk. The model was adjusted for human leukocyte antigen (HLA) risk group, sex, and age at the earliest confirmatory visit, and stratified by study site. As described in the supplement of [13], genotypes from individual studies were harmonized into four HLA risk groups (A, B, C, D), ordered by decreasing risk. Autoantibody positivity indicators for IAA, GADA, IA-2A, at the earliest confirmatory visit, were the primary predictors. A second model analyzed the association between autoantibody titers and diabetes risk. In this model, the log transformed autoantibody titers (log mULN) for IAA, GADA, IA-2A, at the earliest confirmatory visit, were added to the initial model as the primary predictors. The proportional hazards assumption was tested using the
Schoenfeld test [24]. For covariates that did not satisfy the proportional hazards assumption at the 0.05 significance level, time-varying coefficients were used with time modeled linearly [25,26]. P-values corrected for multiple comparisons using the Benjamini-Hochberg method were reported [27].

5. **How well can the autoantibody type-specific titer thresholds identify children with high diabetes risk based on autoantibody screening at different ages?**

Finally, an application of the autoantibody type-specific titer thresholds to identify children at high risk of developing diabetes in the next five years was explored, for potential clinical trial recruitment. Specifically, the ability to use these titer thresholds to stratify diabetes risk (based on results from autoantibody screening at different age ranges), was assessed. The following age ranges were explored: 1-2.0, 2-3.0, 3-4.0, 4-5.0, 5-10.0, and 10+ years. For each age range, children in Study Cohort 1 with at least one autoantibody titer measurement, and without already being diagnosed with diabetes, were included for analysis. These children were stratified based only on the observed autoantibody titers in that age range (when multiple measurements of the same autoantibody were available for a child within the age range, the earliest one was used). Each child was placed into one of twelve possible strata defined by single or multiple autoantibody positivity and the combination of IAA, GADA, and IA-2A titers at-or-above the autoantibody type-specific threshold. KM survival analysis was then performed to estimate the risk of diabetes from the time of the autoantibody measurement, for each age range and each stratum. Children belonging to strata that have an estimated 5-year diabetes risk ≥ 50% were labeled as “high-risk”.

Prediction performance was measured using inverse probability of censoring weighted
(IPCW [28,29]) positive predictive value (PPV), negative predictive value (NPV), sensitivity, and specificity.

Analyses were performed using Python (scikit-learn, scikit-survival) and R (survival, survminer) software [30,31].

**Results**

**Stratification of diabetes risk by islet autoantibody titer quartiles**

Survival analysis from time of confirmed positivity revealed markedly different 5-year diabetes risks associated with IAA (n=909), GADA (n=1076) or IA-2A (n=714), when stratified by quartiles of titer, ranging from 19% (GADA 1st quartile) to 60% (IA-2A 4th quartile) (Supplemental Figure S4). Histogram distributions and quartile thresholds of autoantibody titers, at the confirmatory visit for positivity to IAA, GADA, and IA-2A, are shown in Supplemental Figure S5.

**Determination of autoantibody type-specific titer thresholds that maximize 5-year diabetes risk discrimination**

The results of this analysis for IAA, GADA, and IA-2A are shown in Figure 1 A, B, and C, respectively. The minimum titer value associated with a maximum difference in 5-year diabetes risk differed for each autoantibody type: $T_{\text{IAA}} = 3.6 \, \text{mULN}$, $T_{\text{GADA}} = 5.4 \, \text{mULN}$, and $T_{\text{IA-2A}} = 2.5 \, \text{mULN}$. These titer threshold levels corresponded to 58.6th, 52.4th, and 10.2nd percentile of children positive for IAA, GADA, and IA-2A, respectively.

Using these thresholds, children positive to each autoantibody type were stratified into two groups: those with titers at-or-above threshold and those with titers below threshold. This stratification resulted in significantly different 5-year diabetes risks for all three autoantibody types (all $p<0.0001$) (Supplemental Figure S6).
Improved stratification of diabetes risk by autoantibody type-specific titer thresholds

Stratifying single and multiple islet autoantibody-positive children (determined at time of the earliest confirmatory visit) using the autoantibody type-specific thresholds resulted in significantly different 5-year diabetes risks. For single autoantibody-positive children (n=954), those with antibody titers at-or-above the autoantibody type-specific threshold had a 5-year diabetes risk of 21.9% [95% CI, 17.0-26.4%] (n=364), compared to 6.1% [3.9-8.3%] (n=590) for those with titers below the threshold (Figure 2A). For multiple autoantibody-positive children (n=527), those with zero (n=49), one (n=202), two (n=216), and three (n=60) antibody titers at-or-above the autoantibody type-specific thresholds had a 5-year diabetes risk of 24.7% [95% CI, 10.1-36.9%], 41.2% [33.7-47.9%], 55.7% [48.2-62.1%], and 75.1% [61.0-84.1%], respectively (Figure 2B). The corresponding follow-up times from the earliest confirmatory visit to 50% cumulative progression to diabetes were 8.5yr [95% CI, 7.1-15.0yr], 5.8yr [5.3-6.8yr], 4.0yr [3.3-5.1yr], and 2.3yr [1.6-3.3yr], respectively. A total of 276 children had a 50% or greater risk of developing diabetes within 4 years of first confirmed autoantibody positivity.

Association between islet autoantibody titer and diabetes risk in multivariable analysis

The multivariable regression models that analyzed the association between autoantibodies at the earliest confirmatory visit and diabetes risk are shown in Supplemental Table S3. Model 1 used the autoantibody positivity indicators as predictors. Time-dependent covariates were used for both GADA and IA-2A positivity since they did not satisfy the proportional hazards assumption. At the earliest confirmatory visit, the adjusted HR for GADA positivity was significant (HR 1.43; 95% CI 1.05-1.95; p=0.02) and increased over time (HR 1.06 per year; 95% CI 1.01-1.12; p=0.02). The adjusted HR for IA-2A positivity was significant (HR 3.93; 95% CI 2.95-5.23; p<0.0001) but decreased over time (HR 0.95 per year; 95% CI 0.90-0.99; p=0.02). The adjusted
HR for IAA positivity was also significant (HR 2.10; 95% CI 1.74-2.55; p<0.0001). Age at the earliest confirmatory visit and HLA risk group were also significant. Model 2 adds the corresponding autoantibody titers as predictors. Note that all three of the autoantibody positivity indicators were no longer significant once the autoantibody titers were added. Time dependent covariates were used for IAA titer, since it was the only significant covariate that did not satisfy the proportional hazards assumption. At the earliest confirmatory visit, the adjusted HR for IAA titer was significant (HR 1.37; 95% CI 1.24-1.51; p<0.001) and decreased over time (HR 0.98 per year; 95% CI 0.97-1.0; p =0.01). The adjusted HR for GADA titer (HR1.18; 95% CI 1.11-1.25; p<0.001) and IA-2A titer (HR1.17; 95% CI 1.10-1.24; p< 0.001) were also significant. Age at the earliest confirmatory visit and HLA risk group remained significant. Finally, Model 2 showed higher concordance (standard error) than Model 1: 0.78 (0.01) versus 0.75 (0.01).

**Effectiveness of identifying islet autoantibody-positive children at high risk for diabetes at different ages by using autoantibody type-specific titer thresholds**

The twelve strata, resulting from all possible groupings of single or multiple autoantibody positivity and the combinations of IAA, GADA, IA-2A titers above threshold, and their estimated 5-year diabetes risks are shown in Figure 3 for each age range (the underlying KM analyses are shown in Supplemental Figure S7). Strata that have a 5-year diabetes risk ≥ 50% are considered “high-risk” and are shaded in red. Supplemental Table S4 lists, for each age range, the individual high-risk strata, the “composite high-risk criteria” defined by forming a union of the individual high-risk strata, the total number of children, the number that progressed to diabetes within 5 years, the number of high-risk children identified using the composite high-risk criteria, and the associated PPV, NPV, sensitivity, and specificity performance metrics. There were 167, 289, 231, 283, 60, and 35 high-risk children identified for the age ranges 1-2.0, 2-3.0,
3-4.0, 4-5.0, 5-10.0, and 10+ years, respectively. The PPV was consistent across the age groups ranging from 55% to 65%. Sensitivity ranged from 56% and 74% between ages 1-5 years but dropped significantly to 12% and 14% for ages 5-10 and 10+ years, respectively. As the age of the child being screened increased, not only were more stringent autoantibody criteria needed to identify those with high diabetes risk, but it also became more difficult to reliably identify them. A summary of the process to identify high diabetes risk children is illustrated in the decision flowchart in Figure 4.

Conclusions

This study showed that islet autoantibody titers can stratify risk of progression to diabetes in children, beyond information about the number and type of islet autoantibodies present. Furthermore, these titers matter in different ways for different autoantibodies, and customized islet autoantibody type-specific titer thresholds could be defined that maximized discrimination of the 5-year diabetes risk. The combination of these titer thresholds effectively identified among islet autoantibody-positive children those with a 50% or higher 5-year risk of diabetes who could be potential candidates for participation in intervention trials. The study used data from a large cohort of children prospectively followed in five different birth cohorts, harmonized autoantibody titers across these five studies, and combined them for analysis. Stratification of diabetes risk based on islet autoantibody titer quartiles showed for each of IAA, GADA and IA-2A that higher titers were associated with higher diabetes risk, complementing findings from prior studies [1,3–8]. Islet autoantibodies with high titers usually involve multiple IgG subclasses and are directed against multiple epitopes on the target antigen, likely reflecting a more intense and prolonged autoimmune response and associated with the
progression of diabetes development [12]. The current analysis also revealed that different autoantibody types exhibited different patterns (Supplemental Figure S4). Based on the 5-year diabetes risk, there was no significant separation between neighboring quartiles for IAA, indicating a relatively smooth risk distribution as a function of titer. For GADA, the only significant separation was between the second and third quartiles, indicating a bimodal risk distribution with a gap around the median. For IA-2A, there was only separation between the first and second quartiles, indicating a bimodal risk distribution with a gap around the first quartile. Plots of the cohort percentile as a function of titer threshold at confirmed positivity (Figure 1, top panel) also revealed different distributional behaviors. For IAA, the concave-shaped plot indicated that there were more IAA-positive children with lower IAA titers. The linear-shaped GADA plot indicated an even GADA titer distribution. For IA-2A, the convex-shaped plot indicated that IA-2A-positive children tended to have higher IA-2A titers.

In order to identify islet autoantibody type-specific titer thresholds we used a novel analytical approach that has potential advantages. Specifically, our method was a data-driven approach to automatically identify thresholds that maximize a given outcome. It does this by scanning over the possible threshold values, e.g., increasing autoantibody titers, splitting the cohort based on each threshold value, performing survival analyses on the two resulting groups, and computing the outcome. Using difference in 5-year risk of diabetes as an illustrative outcome of interest, the islet autoantibody type-specific titer thresholds were identified. Furthermore, translation of the islet autoantibody type-specific titer thresholds into percentiles of autoantibody-positive children is important because it allows the thresholds to be applied to external datasets that may have different assay characteristics and normalization methods. The appropriate threshold will depend on the application; notably, the method developed to identify the thresholds is flexible and
generalizable: it can be easily reconfigured to accommodate different outcomes. It may even be adapted for use with other quantified biomarkers such as plasma glucose or glycated hemoglobin. To demonstrate this, we selected a different outcome (e.g., difference in 3-year diabetes risk) and re-ran the analysis. A different set of autoantibody type-specific thresholds were identified that maximized the stratification of 3-year diabetes risk (Supplemental Figure S8) and selected smaller groups of children with higher risk of fast progression to diabetes (Supplemental Figures S9 and S10). It should be noted, however, that because the thresholds were determined based on our study cohort, there may be some uncertainty when extrapolating to other data sets and they may not be as predictive when applied to other cohorts.

The multivariable regression analysis found that the autoantibody positivity indicators for IAA, GADA, IA-2A, at the earliest confirmatory visit, were all significantly associated with diabetes risk. However, when the corresponding autoantibody titers were added, these indicator variables were no longer significant. Instead, all three of the titer variables became significant, indicating that the titers contain more information than the indicators. The HR was time-constant for GADA and IA-2A but decreased over time for IAA. Prior work has found associations between titers and progression to diabetes that were time-constant for IA-2A, but decreased over time for GADA and IAA [11].

The age-based autoantibody screening simulation analysis was able to identify children with a high risk of developing diabetes, using autoantibody positivity and the islet autoantibody type-specific titer thresholds. Of note, the presence of IA-2A above titer threshold alone was sufficient to identify high diabetes risk in children aged 2-5 years, even in the absence of IAA or GADA. It is known that IA-2A usually occurs together with autoantibodies against other beta cell antigens and is therefore highly specific and predictive for progression to clinical diabetes [3,32].
Overall, the results of this study may contribute to improved risk counselling for families of affected children and improved screening for participants for intervention therapy trials aimed at preventing or delaying progression to clinical disease. Since titers add value beyond autoantibody type and number, islet autoantibody standardization programs (e.g., IASP) should continue to focus on improving titer standardization, to facilitate quantitative comparisons across assays and study sites.

This study has some limitations. First, the autoantibody titers were measured using different assays across the study sites. Although the titers were harmonized, some residual biases may remain. In addition, the current data are based on radio binding assay results. Islet autoantibody type-specific titer thresholds and respective percentiles of positives may need to be adjusted for other assay formats such as those based on electrochemiluminescence [33], luciferase immunoprecipitation system [34], or agglutination-PCR [35] technology. Second, due to differences in the visit intervals of the study protocols, it is possible that the actual time of the earliest autoantibody positivity was missed, with the consequence that the measured time is biased. Off-schedule visits may also impact the timing of the confirmatory visit. Third, only children with increased genetic and familial risk for development of islet autoimmunity and diabetes were enrolled into the studies, and the study populations were predominantly Caucasian, which may limit generalizability of the results. Fourth, the analyses have not been validated on an independent cohort.

There are several possible directions for future work. First, the analyses should be replicated in higher time resolution datasets with more frequent prospective follow-up (e.g., TEDDY [36]). Second, validation in independent cohorts with broader population inclusion criteria (e.g., Fr1da [37] or ASK studies [38]) should be undertaken. Third, the age-based risk stratification
performance should be validated in a cross-sectional study. Fourth, the utility of islet autoantibody titers as a continuous variable should be further explored in diabetes risk prediction [39] and disease progression modeling [40].

In summary, this study harmonized islet autoantibody titers across multiple birth cohorts, combined them for analysis, and defined autoantibody type-specific titer thresholds that significantly improved type 1 diabetes risk stratification in children.
Acknowledgments

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**Author contributions**: KN, PA, WH conceived and designed the study. KN, HS, VA, RV, JT, MM, KW, WH, PA acquired, analyzed, and interpreted the data. KN, PA drafted the manuscript. KN, HS, VA, RV, JT, MM, ML, KW, BF, FM, WH, PA critically revised the manuscript for important intellectual content. All authors gave final approval of the version to be submitted.
References


**Figure Captions**

**Figure 1:** Identifying autoantibody type-specific titer thresholds for IAA (A), GADA (B), and IA-2A (C). Top panel: The size of the red cohort (titer ≥ threshold) and the green cohort (titer < threshold) for each autoantibody titer threshold level. Middle panel: 5-year risk of diabetes and 95% confidence intervals from the time of the confirmatory visit for autoantibody positivity for the red and green cohorts for each titer threshold level. Bottom panel: The difference in the 5-year diabetes risk between the red and green cohorts for each titer threshold level. An arrow marks the lowest titer threshold level where there is a maximum risk difference between the cohorts and the threshold covers up to 75% of the cohort (T_{IAA} = 3.6 mULN, T_{GADA} = 5.4 mULN, and T_{IA-2A} = 2.5 mULN). The percentile of children who tested positive for the respective autoantibody corresponding to the final titer threshold is highlighted in the top panel (T_{IAA} → 58.6%, T_{GADA} → 52.4%, and T_{IA-2A} → 10.2%). IAA indicates insulin autoantibodies; GADA, glutamic acid decarboxylase autoantibodies; IA-2A, insulinoma-associated antigen-2 autoantibodies; mULN, multiples of upper limit of normal; DM, diabetes mellitus.

**Figure 2:** Progression to diabetes from the time of the earliest confirmatory visit in children with single and multiple autoantibody positivity. Stratification is based on the autoantibody titer measured at the earliest confirmatory visit and the identified autoantibody type-specific titer thresholds (T_{IAA} = 3.6 mULN, T_{GADA} = 5.4 mULN, T_{IA-2A} = 2.5 mULN). (A) Single autoantibody-positive children are partitioned into two groups: those with autoantibody titer below threshold (t < T) and those with titer at-or-above threshold (t ≥ T). (B) Multiple autoantibody-positive children are partitioned into four mutually exclusive groups: those with no autoantibody titer at-or-above threshold (0IAb ≥ T), those with one autoantibody titer at-or-above threshold (1IAb ≥ T), those with two autoantibody titers at-or-above threshold (2IAb ≥ T), and those with all three autoantibody titers at-or-above threshold (3IAb ≥ T). The dashed vertical line marks the 5-year follow-up time point. IAA indicates insulin autoantibodies; GADA, glutamic acid decarboxylase autoantibodies; IA-2A, insulinoma-associated antigen-2 autoantibodies; mULN, multiples of upper limit of normal.
Figure 3: The 5-year risk of type 1 diabetes and 95% confidence intervals in subjects who developed confirmed-positive islet autoantibodies, stratified by single or multiple autoantibody positivity and the combination of IAA, GADA, IA-2A titers above thresholds (T_{IAA} = 3.6 mULN, T_{GADA} = 5.4, mULN T_{IA-2A} = 2.5 mULN) for screening at different age ranges (A: 1-2.0 y, B: 2-3.0 y, C: 3-4.0 y, D: 4-5.0 y, E: 5-10.0 y, F: 10+ y). The 12 strata are:

- S:0T:-- = single positive, no autoantibodies above titer threshold
- S:1T:GADA = single positive, one (GADA) above titer threshold
- S:1T:IAA = single positive, one (IAA) above titer threshold
- S:1T:IA-2A = single positive, one (IA-2A) above titer threshold
- M:0T:-- = multiple positive, no autoantibodies above titer threshold
- M:1T:GADA = multiple positive, one (GADA) above titer threshold
- M:1T:IAA = multiple positive, one (IAA) above titer threshold
- M:1T:IA-2A = multiple positive, one (IA-2A) above titer threshold
- M:2T:GADA,IAA = multiple positive, two (GADA, IAA) above titer threshold
- M:2T:GADA,IA-2A = multiple positive, two (GADA, IA-2A) above titer threshold
- M:2T:IA-2A,IAA = multiple positive, two (IA-2A, IAA) above titer threshold
- M:3T:GADA,IA-2A,IAA = multiple positive, all three above titer threshold

The number of subjects in each stratum is shown at the base of each bar. The dashed vertical red lines mark the 50% 5-year risk of diabetes level. Strata that exceed that risk level are classified as “high-risk” and shaded red. IAA indicates insulin autoantibodies; GADA, glutamic acid decarboxylase autoantibodies; IA-2A, insulinoma-associated antigen-2 autoantibodies; mULN, multiples of upper limit of normal.

Figure 4: A proposed flowchart to discover islet autoantibody-positive children and then evaluate their antibody titer to identify those at high risk (≥50%) of developing type 1 diabetes within 5 years. A child can enter the flowchart by autoantibody testing at any age via a blue arrow and appropriate blue box. Those with antibodies fulfilling the titer criteria shown in the corresponding grey box are at high risk and could be considered for intervention therapy trials or close glycemic monitoring. Islet autoantibodies tested include insulin autoantibodies (IAA), glutamic acid decarboxylase autoantibodies (GADA), and insulinoma-associated antigen-2 autoantibodies (IA-2A).
A

Risk of Diabetes vs IAA Titer (n=909)

 absenteeism 58.6%

IAA Titer (mULN) @ confirmatory visit

Risk of Diabetes vs GADA Titer (n=1076)

 GADA Titer (mULN) @ confirmatory visit

Risk of Diabetes vs IA-2A Titer (n=714)

 IA-2A Titer (mULN) @ confirmatory visit

TIAA = 3.6

TGADA = 5.4

TIAA-2A = 2.5
Supplementary Appendix

Supplement to Ng et al., Islet autoantibody type-specific titer thresholds improve stratification of risk of progression to type 1 diabetes in children

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**Supplemental Table S1**: Key characteristics of the Study Cohort 1 population.

Plus–minus values are means ±SD. Percentages may not total to 100 because of rounding. Autoantibody-positive percentages may not total to 100 due to multiple positivity.

Abbreviations: HLA, human leukocyte antigen; GADA, glutamic acid decarboxylase autoantibodies; IAA, insulin autoantibodies; IA-2A, insulinoma-associated antigen-2 autoantibodies; mULN, multiples of upper limit of normal.

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<td>IA-2A Initial Visit</td>
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<td>GADA Confirmatory Visit</td>
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<td>IA-2A</td>
<td>90.7 ±145.7</td>
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### Supplemental Table S2: Key characteristics of the Study Cohort 2 population.

Plus–minus values are means ±SD. Percentages may not total to 100 because of rounding. Autoantibody-positive percentages may not total to 100 due to multiple positivity. Abbreviations: HLA, human leukocyte antigen; GADA, glutamic acid decarboxylase autoantibodies; IAA, insulin autoantibodies; IA-2A, insulinoma-associated antigen-2 autoantibodies; mULN, multiples of upper limit of normal.

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<tr>
<td>Earliest autoantibody positivity (confirmatory visit)</td>
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<td>Min-Max age at earliest positivity (confirmatory visit)</td>
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<td>DAISY</td>
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<td>DEW-IT</td>
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<td>DIPIS</td>
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<td>IA-2A</td>
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<td>IA-2A</td>
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Supplemental Figure S1: Study cohort selection flowchart. IAA indicates insulin autoantibodies; GADA, glutamic acid decarboxylase autoantibodies; and IA-2A, insulinoma-associated antigen-2 autoantibodies.
Supplemental Figure S2: Distributions of the raw (in original assay-specific units) and normalized (in multiples of upper limit of normal – mULN – units) titer levels from the confirmatory visit for positivity to IAA (A), GADA (B), and IA-2A (C), across the five studies. BABYDIAB reported high IA-2A levels outside standard curve as 201 units. IAA indicates insulin autoantibodies; GADA, glutamic acid decarboxylase autoantibodies; and IA-2A, insulinoma-associated antigen-2 autoantibodies.
Supplemental Figure S3: Illustration of the relative temporal relationships between the initial visit for autoantibody positivity, the confirmatory visit for autoantibody positivity (time 0), and the outcome event: either type 1 diabetes diagnosis or end of follow up (censored).
Supplemental Figure S4: Progression to diabetes from the time of the confirmatory visit for positivity of IAA (A), GADA (B), and IA-2A (C). Stratification is based on quartiles of autoantibody-positive values, at the confirmatory visit for positivity to the specific autoantibody. The dashed vertical line marks the 5-year follow-up time point. (D) The 5-year diabetes risk estimates and 95% CIs for the quartile strata for each of the three autoantibody types. IAA indicates insulin autoantibodies; GADA, glutamic acid decarboxylase autoantibodies; and IA-2A, insulinoma-associated antigen-2 autoantibodies.
Supplemental Figure S5: Histogram distributions and quartile thresholds of autoantibody titer levels, at the confirmatory visit for positivity to IAA (A), GADA (B), and IA-2A (C). IAA indicates insulin autoantibodies; GADA, glutamic acid decarboxylase autoantibodies; IA-2A, insulinoma-associated antigen-2 autoantibodies; mULN, multiples of upper limit of normal.
Supplemental Figure S6: Progression to diabetes from the time of the confirmatory visit for positivity, to IAA (A), GADA (B), and IA-2A (C). Stratification is based on the autoantibody-specific thresholds (T_{IAA} = 3.6 mULN, T_{GADA} = 5.4 mULN, and T_{IA-2A} = 2.5 mULN) applied to the autoantibody titer levels from the confirmatory visit for positivity to the specific autoantibody. The dashed vertical line marks the 5-year follow-up time point. (D) The 5-year diabetes risk estimates and 95% CIs for the below threshold (<T) and at/above threshold (>=T) strata for each of the three autoantibody types. IAA indicates insulin autoantibodies; GADA, glutamic acid decarboxylase autoantibodies; IA-2A, insulinoma-associated antigen-2 autoantibodies; mULN, multiples of upper limit of normal.
Supplemental Table S3: Multivariable Cox proportional hazards regression models to analyze the association between autoantibodies at the earliest confirmatory visit and type 1 diabetes risk. The regression formula, variables, coefficients, hazard ratios, 95% confidence intervals, P-values, significance indicators, and concordance (standard error) from the fitted models are shown. The models were adjusted for HLA risk group, sex, age at the earliest confirmatory visit and stratified by study site. Model 1 uses the autoantibody positivity indicators for IAA, GADA, IA-2A, at the earliest confirmatory visit, as the primary predictors. Time varying coefficients for GADA positivity and IA-2A positivity were used to handle violations of the proportional hazard assumption (assessed via the Schoenfeld test). Model 2 adds log normalized autoantibody titers for IAA, GADA, IA-2A, at the earliest confirmatory visit to Model 1 as the primary predictors. Time varying coefficients for IAA titer were used to handle violation of the proportional hazard assumption. The \(tt(\ldots)\) function indicates the constructed time dependent covariates which are interactions of the predictor and survival time used for estimating the time varying coefficients.
Supplemental Figure S7: The risk of type 1 diabetes in subjects who developed confirmed-positive islet autoantibodies, stratified by single or multiple autoantibody positivity and the combination of IAA, GADA, IA-2A titers above thresholds (T_{IAA} = 3.6 mULN (multiples of upper limit of normal), T_{GADA} = 5.4 mULN, T_{IA-2A} = 2.5 mULN) for screening at different age ranges. The strata are:

- S:0T:-- = single positive, no autoantibodies above titer threshold
- S:1T:GADA = single positive, one (GADA) above titer threshold
- S:1T:IAA = single positive, one (IAA) above titer threshold
- S:1T:IA-2A = single positive, one (IA-2A) above titer threshold
- M:0T:-- = multiple positive, no autoantibodies above titer threshold
- M:1T:GADA = multiple positive, one (GADA) above titer threshold
- M:1T:IAA = multiple positive, one (IAA) above titer threshold
- M:1T:IA-2A = multiple positive, one (IA-2A) above titer threshold
- M:2T:GADA,IAA = multiple positive, two (GADA, IAA) above titer threshold
- M:2T:GADA,IA-2A = multiple positive, two (GADA, IA-2A) above titer threshold
- M:2T:IA-2A,IAA = multiple positive, two (IA-2A, IAA) above titer threshold
- M:3T:GADA,IA-2A,IAA = multiple positive, all three above titer threshold
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<td>189</td>
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<td>207</td>
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<td>IA-2A ≥ specific threshold titer</td>
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<td>74.3%</td>
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<td>157</td>
<td>62.3%</td>
<td>80.5%</td>
<td>11.8%</td>
<td>98.1%</td>
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</table>

**Supplemental Table S4**: Description and performance of the high diabetes risk strata.

For each age range group, the following information is shown:

- All strata with 5-year diabetes risk >= 50%.
- The composite high-risk criteria defined by combining the criteria of the separate strata.
- The total number of children, number that progressed to diabetes within 5 years, and the number of high-risk children identified using the high diabetes risk stratum.
- The inverse probability of censoring weighted (IPCW) positive predictive value, negative predictive value, sensitivity, and specificity.

IAA indicates insulin autoantibodies; GADA, glutamic acid decarboxylase autoantibodies; and IA-2A, insulinoma-associated antigen-2 autoantibodies.
Supplemental Figure S8: Identifying autoantibody type-specific titer thresholds for IAA (A), GADA (B), and IA-2A (C) using a 3-year risk of diabetes outcome. Top panel: The size of the red cohort (titer ≥ threshold) and the green cohort (titer < threshold) for each autoantibody titer threshold level. Middle panel: 3-year risk of diabetes and 95% confidence intervals from the time of the confirmatory visit for autoantibody positivity for the red and green cohorts for each titer threshold level. Bottom panel: The difference in the 3-year diabetes risk between the red and green cohorts for each titer threshold level. An arrow marks the lowest titer threshold level where there is a maximum risk difference between the cohorts and the threshold covers up to 75% of the cohort (T_{IAA} = 5.8 mULN, T_{GADA} = 6.3 mULN, and T_{IA-2A} = 2.5 mULN). The percentile of children who tested positive for the respective autoantibody corresponding to the final titer threshold is highlighted in the top panel (T_{IAA} → 73.9%, T_{GADA} → 55.9%, and T_{IA-2A} → 10.2%). IAA indicates insulin autoantibodies; GADA, glutamic acid decarboxylase autoantibodies; IA-2A, insulinoma-associated antigen-2 autoantibodies; mULN, multiples of upper limit of normal; DM, diabetes mellitus.
Supplemental Figure S9: Progression to diabetes from the time of the earliest confirmatory visit in children with single and multiple autoantibody positivity. Stratification is based on the autoantibody titer measured at the earliest confirmatory visit and the identified autoantibody type-specific titer thresholds ($T_{IAA}=5.8$ mULN, $T_{GADA}=6.3$ mULN, $T_{IA-2A}=2.5$ mULN) from Supplemental Figure S8. (A) Single autoantibody-positive children are partitioned into two groups: those with autoantibody titer below threshold ($t < T$) and those with titer at-or-above threshold ($t \geq T$). (B) Multiple autoantibody-positive children are partitioned into four mutually exclusive groups: those with no autoantibody titer at-or-above threshold ($0IAb \geq T$), those with one autoantibody titer at-or-above threshold ($1IAb \geq T$), those with two autoantibody titers at-or-above threshold ($2IAb \geq T$), and those with all three autoantibody titers at-or-above threshold ($3IAb \geq T$). The dashed vertical line marks the 3-year follow-up time point. IAA indicates insulin autoantibodies; GADA, glutamic acid decarboxylase autoantibodies; IA-2A, insulinoma-associated antigen-2 autoantibodies; mULN, multiples of upper limit of normal.
Supplemental Figure S10: The 3-year risk of type 1 diabetes and 95% confidence intervals in subjects who developed confirmed-positive islet autoantibodies, stratified by single or multiple autoantibody positivity and the combination of IAA, GADA, IA-2A titers above thresholds (T_{IAA} = 5.8 \text{ mULN}, T_{GADA} = 6.3 \text{ mULN}, T_{IA-2A} = 2.5 \text{ mULN}) for screening at different age ranges (A: 1-2.0y, B: 2-3.0y, C: 3-4.0y, D: 4-5.0y, E: 5-10.0y, F: 10+ y). The 12 strata are the same as those described in Figure 3. The number of subjects in each stratum is shown at the base of each bar. The dashed vertical red lines mark the 50% 3-year risk of diabetes level. Strata that exceed that risk level are classified as “high-risk” and shaded red. IAA indicates insulin autoantibodies; GADA, glutamic acid decarboxylase autoantibodies; IA-2A, insulinoma-associated antigen-2 autoantibodies; mULN, multiples of upper limit of normal.
Section S1: Islet autoantibody titer normalization

As summarized in the supplement for [1], each study used different assays to measure the islet autoantibodies: IAA, GADA, and IA-2A. The threshold for positivity (i.e., the upper limit of normal) is assay dependent and was determined by each study, usually as the 99th percentile of their normal, healthy, nondiabetic, control test subject population. In BABYDIAB, very high IA-2A values that were outside the standard curve (>200 units) were reported as 201 units in years 2001-2009. Each of the studies employed rigorous quality control procedures to control for drift in the assays, and their laboratories have participated with satisfactory results in all proficiency workshops of the Diabetes Autoantibody Standardization program (DASP) [2–4] and the Islet Autoantibody Standardization program (IASP) [5]. The results of these workshops demonstrated that the different laboratories had excellent discrimination between type 1 diabetic and control sera, high sensitivities, and high specificities. More importantly, the results demonstrated good concordance between the different laboratories in the ranking of samples by IAA, GADA, and IA-2A levels (which is an important prerequisite to be able to compare titers across studies).

The different laboratory assays report autoantibody titer measurements in terms of either “indices” or “arbitrary/relative units.” Index titers are computed based on negative and positive control samples using the formula:

\[ t_i = \frac{(t_\text{s} - t_-)}{(t_+ - t_-)} \]

where \( t_\text{s} \) is the (mean) titer measurement of the unknown subject sample, \( t_+ \) is the (mean) titer measurement of the positive control sample, and \( t_- \) is the (mean) titer measurement of the negative control sample. The original titer measurements are usually in cpm (counts per minute) or od (optical density).
Arbitrary or relative units were computed using several methods [3]. One approach uses a formula based on one reference standard sample, and is very similar to the index formula above:

\[ t_r = N \frac{(t_s - t_-)}{(t_+ - t_-)} \]

where \( t_s \) is the (mean) titer measurement of the unknown subject sample, \( t_+ \) the (mean) titer measurement of the positive control sample, \( t_- \) the (mean) titer measurement of the negative control sample, and \( N \) a constant used to scale the units relative to a positive reference with an arbitrary value of \( N \) units. Another approach uses a “standard curve,” based on multiple standard samples by constructing a regression curve for the titer measurement (cpm or od) versus the assigned reference in units/ml, for each of the known standard samples. This regression curve can then be used to convert the titer measurement of the unknown samples (in cpm or od) into the desired reference relative units (in units/ml):

\[ t_r = at_s + b \]

where \( t_s \) is the (mean) titer measurement of the unknown subject sample, \( a \) the slope, and \( b \) the intercept (i.e., \( t_r = b \) when \( t_s = 0 \)) of the fitted regression.

Since the titer measurements for the same autoantibody, from different assays, with different index and relative units, are not directly comparable, we converted the autoantibody titer measurements into multiples of upper limit of normal (mULN), by dividing the subject titer value, \( t \), by the positivity threshold level, \( T \), for the corresponding assay:

\[ t_{mULN} = \frac{t}{T} \]

Positive autoantibody test results will have a value \( \geq 1 \) and negative autoantibody test results will have a value \( < 1 \). For a given assay, both \( t \) and \( T \) are in the same units. Taking the ratio of the two quantities removes some of the underlying variations across assays and allows us to compare the titers. In the case of index units \( (t_I, T_I) \), we have:
\[ t_{mULN} = \frac{t_i}{T_i} = \frac{(t_s - t_-)/(t_+ - t_-)}{(T - t_-)/(t_+ - t_-)} = \frac{(t_s - t_-)}{(T - t_-)} \]

In the denominator, \( T \gg t_- \), since the positivity threshold \( T \), computed as the upper limit of normal or 99th percentile of the normal control test subject population, will be much larger than \( t_- \), the (mean) titer measurement of the negative control sample (which will be small and around the 50th percentile (median) of the normal control test subject population). In the numerator, for subjects that are autoantibody positive (which is the case that we are interested in), \( t_s \geq T \), and, as a result, \( t_s \gg t_- \). In this case, we can approximate \( t_{mULN} \) as:

\[ t_{mULN} \approx \frac{t_s}{T} \]

In the case of relative units \((t_r, T_r)\) using a single standard, we have:

\[ t_{mULN} = \frac{t_r}{T_r} = \frac{N(t_s - t_-)/(t_+ - t_-)}{N(T - t_-)/(t_+ - t_-)} = \frac{(t_s - t_-)}{(T - t_-)} \]

Again, with \( T \gg t_- \) and \( t_s \gg t_- \), when the subject is autoantibody positive, we have:

\[ t_{mULN} \approx \frac{t_s}{T} \]

For relative units \((t_r, T_r)\) derived using a standard curve from multiple standards, we have:

\[ t_{mULN} = \frac{t_r}{T_r} = \frac{a t_s + b}{a T + b} \]

Since \( t_r = b \) when \( t_s = 0 \), we expect \( b \) to be a small value, especially when compared to the values of \( T \) and \( t_s \), when the subject is autoantibody positive. In this case, we have \( T \gg b \) and \( t_s \gg b \), and can approximate \( t_{mULN} \) as:

\[ t_{mULN} \approx \frac{a t_s}{a T} = \frac{t_s}{T} \]

Although not perfect, by converting the autoantibody titers into multiples of upper limit of normal (mULN), we obtain measurements that are more comparable for our use case
(autoantibody positivity) and given reasonable assumptions (the values of $b$ and $t_-$ are small and near 0).

Distributions of the raw (in original assay-specific index or arbitrary units) and normalized (in mULN units) titer levels, from the confirmatory visit for positivity to IAA, GADA, and IA-2A for Study Cohort 1 (Table 1), are shown in Supplemental Figure S2. With the original raw titer levels, there is a large difference in the dynamic range, and there is little overlap across study sites. However, with the mULN normalized titer levels, there is a much narrower dynamic range, and more significant overlap across study sites.
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References


