Pancreatic Islet Autoantibodies as Predictors of Type 1 Diabetes in the Diabetes Prevention Trial-Type 1 (DPT-1)

Running Title: Type 1 Diabetes Prediction by Autoantibodies

Tihamer Orban, MD (1); Jay M. Sosenko, MD (2); David Cuthbertson, MS (3); Jeffrey P. Krischer, PhD (4); Jay S. Skyler, MD (2); Richard Jackson (1); Liping Yu, MD (5); Jerry P. Palmer, MD (6); Desmond Schatz, MD (7); George Eisenbarth, MD, PhD (5); Diabetes Prevention Trial-1Study Group (1)

(1) Joslin Diabetes Center, Boston, Massachusetts;
(2) Division of Endocrinology, University of Miami, Miami, FL;
(3) Pediatrics Epidemiology Center, University of South Florida, Tampa, Florida;
(4) Division of Informatics and Biostatistics, University of South Florida, Tampa, Florida;
(5) Barbara Davis Center for Childhood Diabetes, Denver, Colorado;
(6) Division of Endocrinology/ Metabolism, University of Washington, Seattle, Washington;
(7) Division of Endocrinology, University of Florida, Gainesville, FL

Corresponding Author:
Jay M. Sosenko, MD (2)
Email: jsosenko@med.miami.edu

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Objective. There is limited information from large-scale prospective studies regarding the prediction of type 1 diabetes (T1D) by specific types of pancreatic islet autoantibodies, either alone or in combination. Thus, we studied the extent to which specific autoantibodies are predictive of T1D.

Research Design and Methods. Two cohorts were derived from the first screening for islet cell autoantibodies (ICA) in the Diabetes Prevention Trial-1 (DPT-1). Autoantibodies to glutamic acid decarboxylase 65 (GAD65), insulinoma associated antigen-2 (ICA512), and insulin (micro IAA or mIAA) were also measured. Participants were followed for the occurrence of T1D. One cohort (Questionnaire) included those who did not enter the DPT-1 trials, but responded to questionnaires (n=28,507, 2.4% ICA positive). The other cohort (Trials) included DPT-1 trial participants (n=528, 83.3% ICA positive).

Results. In both cohorts autoantibody number was highly predictive of T1D (p<0.001). The Questionnaire cohort was utilized to assess prediction according to the type of autoantibody. As single autoantibodies, ICA (3.9%), GAD65 (4.4%) and ICA512 (4.6%) were similarly predictive of T1D in proportional hazards models (p<0.001 for all). However, none with mIAA as single autoantibodies developed T1D. As second autoantibodies, all except mIAA added significantly (p<0.001) to the prediction of T1D. Within the positive range, GAD65 and ICA autoantibody titers were predictive of T1D.

Conclusions. The data indicate that the number of autoantibodies is predictive of T1D. However, mIAA is less predictive of T1D than other autoantibodies. Autoantibody number, type of autoantibody and autoantibody titer must be carefully considered in planning prevention trials for T1D.
Autoantibodies to islet cell antigens are known predictors of type 1 diabetes (T1D) and are commonly present at its diagnosis (1-12). Islet-cell autoantibodies (ICA), the first identified (1,2), actually represent autoimmunity to several different antigens. More recently, autoantibodies specific to single tissue antigens, termed biochemical autoantibodies, have been identified (4,7,8,11-13). These include antibodies to glutamic acid decarboxylase 65 (GAD65), the antibody to an insulinoma associated antigen-2 (ICA512), and antibodies to insulin (IAA).

T1D prevention trials have utilized autoantibodies to screen for individuals at increased risk who might be candidates for participation (14-16). The Diabetes Prevention Trial-Type 1 (DPT-1) assessed parenteral and oral insulin as potential prevention modalities. First and second-degree relatives of T1D patients were screened for the presence of ICA, which was required for eligibility. Although not relevant to the trials, biochemical autoantibodies were subsequently measured from screening samples in order to learn more about their prediction of T1D. The prevalence of autoantibodies according to various subgroups has been reported for DPT-1 (17).

We utilized two DPT-1 cohorts to examine the prediction of T1D by ICA and biochemical autoantibodies, since few large-scale studies have examined the prediction of T1D by a variety of single autoantibodies in large numbers of individuals of whom many ultimately developed T1D. One cohort includes DPT-1 participants who participated in the trials (the Trials cohort), while the other cohort includes participants who did not participate in either trial, but responded to questionnaires (the Questionnaire cohort) used to ascertain information regarding the diagnosis of T1D. The differing perspectives of these two cohorts and the large number of individuals studied, almost 30,000, provide a unique opportunity for studying the prediction of T1D by autoantibodies.

RESEARCH DESIGN AND METHODS

Subjects. All participants were relatives of patients with T1D. There were 97,273 serum samples collected and tested for ICA at the initial screening. Informed consent was obtained from all subjects. As described elsewhere (14,15), eligibility for the trials was further assessed on the basis of metabolic abnormalities (parenteral insulin trial) and the presence of IAA (oral insulin trial). There were 711 individuals who participated in the DPT-1 trials. Of the screening samples, 84% were later tested for the presence of GAD65, ICA512 and IAA measured by the micro method (mIAA).

Questionnaires were mailed to 79,292 who did not enter the trials. Those who were ICA positive did not meet criteria for trial entry or chose not to enter the trials. Responses were received from 37,017 subjects. Those who had all autoantibody determinations and sufficiently complete data were included in the analyses (n=29,035).

Procedures: Questionnaire Cohort -- Participants were asked whether they were informed by a physician that they had developed T1D. If participants answered affirmatively, they were asked when they were diagnosed. The follow-up interval was the time between the date of the response to the questionnaire and the date of the initial screen for autoantibodies (those who did not develop T1D) or between the date of diagnosis as indicated on the questionnaire and the date of the initial screen (those who developed T1D). The mean±SD age of the individuals in the Questionnaire cohort (n=28,507) was 17.9±13.0 years (55% female). The duration of follow-up was 4.2±2.4 years.
Trials Cohort--The procedures for the DPT-1 trials have been described elsewhere (14,15). In both the parenteral and oral insulin trials, OGTTs were scheduled for six month intervals. Blood samples were obtained for plasma glucose and C-peptide measurements in the fasting state and at 30, 60, 90 and 120 minutes. Those with glucose values in the diabetic range (fasting glucose $\geq 126$ mg/dl and/or 2-hr glucose $\geq 200$ mg/dl) were asked to return for confirmation at a follow-up visit. The follow-up interval was the time between the date of last contact and the date of the first screen (those who did not develop T1D) or the time between the date of diagnosis and the date of the first screen (those who developed T1D). Of those diagnosed with T1D in the DPT-1 trials, 61% were diagnosed at a routine visit. The others were diagnosed clinically. There was no overall effect of the intervention in either trial (14,15). The mean±SD age of those in the Trials cohort (n=528) was 12.2±9.3 years (43% female). The Trials cohort was significantly younger and had a lower proportion of females than the Questionnaire cohort (p<0.001 for both). The duration of follow-up was 4.4±2.2 years.

Laboratory Measures: ICA--ICA was determined by an immunofluorescence assay on frozen sections of blood type O human pancreas in the DPT-1 ICA Core Laboratory (Gainesville, FL, February 1994 to September 1997 and January 1999 to October 2003; New Orleans, LA, September 1997 to January, 1999). ICA values of 10 or more Juvenile Diabetes Foundation (JDF) units were considered positive. In the 1995 Immunology of Diabetes Society (IDS) workshop (18), this ICA assay had a specificity of 100% and a sensitivity of 74.4% for new-onset type 1 diabetes patients less than 30 years of age. Based on a receiver-operator characteristics curve (ROC), in this data set with a positive JDF value of 10, the assay sensitivity was 75.0% with a 95.7% specificity (no age influence on the values).

GAD65, ICA512 and mIAA--Autoantibodies against glutamic acid decarboxylase-65 (GAD65) and ICA512bdc (ICA512) were determined at the Barbara Davis Center (Denver, CO). Insulin autoantibodies (using the micro-volume requiring assay) were determined at the Barbara Davis Center or the Joslin Diabetes Center (Boston, MA). As previously described, a combined GAD65 and ICA512 radioassay was performed (19). Labeled recombinant GAD65 and ICA512 were produced by in vitro transcription/translation with differential labeling ([1H]GAD65 and [35S]ICA512) (8,13). The levels of both autoantibodies were expressed as an index. The upper limits of normal (0.032 for GAD65; 0.049 for ICA512) were established as the 99th percentile for GAD65 and for ICA512 from receiver-operator characteristics curves in 198 healthy control subjects and 50 patients with new-onset diabetes. In this data set a GAD65 index of 0.032 (used for the analysis) provided a 41.8% sensitivity and a 98.3% specificity (no difference by age group), and for ICA512 an index of 0.049 (used for the analysis) resulted in a sensitivity of 57.5% and specificity of 98.5% (no difference by age group). In the 2000, 2002 and the 2003 Diabetes Antibody Standardization Programs (DASP) for proficiency testing, the sensitivity/specificity results for the GAD65 assay were 84%/96%, 90%/93%, 84%/98%, and for the ICA512 assay, 52%/100%, 62%/99%, 58%/100%, respectively. The interassay coefficients of variation for ICA512 and GAD65 were 8% and 10%, respectively.

The mIAA assay (20) was performed as described and expressed as an index with the upper limits of 0.02 and 0.01 (Boston and Denver laboratories, respectively) based on the 99th percentile of healthy control values. The two laboratories measured a similar number of samples, and the combined data set with a positive index value had 36.8%
sensitivity and 92.7% specificity. Determination of mIAA on samples began later than for GAD65 and ICA512 (assay development needed). In the 2003 Diabetes Autoantibody Standardization Program (DASP) proficiency testing, the sensitivity for the mIAA assay was 74% and 56%, and the specificity was 90% and 98%, respectively, for the Denver and Boston laboratories. The correlation coefficient between both laboratories was $r=0.90$, $p<0.0001$. The interassay coefficient of variation for mIAA was 16%.

**Data Analysis.** T-tests and chi-square tests were utilized to compare groups. The log-rank test was used to compare the distributions of event-times between groups. Cox Proportional Hazards regression models were employed to examine effects on T1D risk over time. The Kaplan-Meier estimate of the survival function was used to obtain estimates of T1D occurrence. Spearman correlations were performed to assess associations. Log transformations were performed for certain analyses.

The SAS 9.1.3 version was used for the analyses. All p-values are 2-sided. The level of significance was $p<0.05$.

**RESULTS**

The prevalence for each autoantibody at the initial screening is shown in Table 1 for both cohorts. Autoantibody prevalence was much higher in the Trials cohort, which is attributable to the selection for ICA positivity and for the additional trial entry criteria. Although some of those in the Trials cohort were negative for ICA at the initial screening, all eventually became ICA positive prior to randomization.

In the Questionnaire cohort, ICA512 positivity was most commonly associated with one or more autoantibodies (65%), while mIAA positivity was least commonly associated with other autoantibodies (22%). The percentages of GAD65 and ICA positivity with associated autoantibodies were 37% and 39%, respectively.

The occurrence of T1D according to the number of biochemical autoantibodies is shown separately for the Questionnaire and Trials cohorts and in the aggregate in Figure 1. In each cohort there tended to be an increasing occurrence of T1D as the number of autoantibodies increased ($p<0.001$ for both).

The occurrence of T1D among those with a single autoantibody is shown in Table 2 for the Questionnaire cohort. None of the 407 with the presence of only mIAA developed T1D. The occurrence of T1D was similar for those with GAD65 alone (4.4%), ICA512 alone (4.6%) and ICA alone (3.9%). In proportional hazards models there were significant associations between the occurrence of T1D and each of those autoantibodies occurring singly ($p<0.001$ for all). When age was added as a covariate, the associations remained highly significant ($p<0.001$).

Supplemental Table A1 shows the occurrence of T1D among those who were single autoantibody positive in the combined Questionnaire and Trials cohorts (see Online Appendix available at [http://care.diabetesjournals.org](http://care.diabetesjournals.org)). The occurrence of T1D for those with ICA alone was somewhat higher than the occurrence for the other autoantibodies alone.

The distribution of mIAA values was examined to determine whether the lack of occurrence of T1D in those with mIAA alone could be the result of a preponderance of low titers. Since mIAA was measured in two laboratories (positives: $n=123$ for Boston and $n=284$ for Denver) and the threshold was higher for an abnormal value in Boston than in Denver (0.02 vs. 0.01), the distributions were examined for each lab. Among those with abnormal values, the median values for Boston and Denver were 0.108 and 0.024, respectively. The values for the 75th
percentiles were 0.182 and 0.051, respectively. Thus, an appreciable proportion of the titers was clearly elevated.

Figure 2 shows the effect of adding each autoantibody as a second autoantibody to the presence of a single autoantibody (any of the other three). When ICA, ICA512 and GAD65 was each included as a second autoantibody, the occurrence of T1D was significantly greater (p<0.001 for each) than when there was single autoantibody positivity. However, with mIAA as a second autoantibody there was no significant difference from the presence of one autoantibody. [There was little difference in risk increment when either GAD65 or ICA was the first autoantibody and the other was added (data not shown.)] When each of the autoantibodies was present additionally as a third autoantibody (Supplemental Figure A1 in the Online Appendix), the risk increased appreciably with ICA and ICA512 (both p<0.001), but did not increase significantly with GAD65 and mIAA.

The Trials cohort was utilized to assess the influence of a second autoantibody besides ICA. There was an increase in the percentage of those developing T1D when GAD65 and ICA512 each was present besides ICA [ICA alone: 13/65 (20%); ICA with GAD65: 30/87 (34%); ICA with ICA512: 11/22 (50%)]. However, when age was included as a covariate in proportional hazards models, neither the additional presence of GAD65 nor that of ICA512 was significant. The numbers for the additional presence of mIAA as a second autoantibody were too small for a meaningful analysis; however, 3/6 (50%) developed T1D.

Associations of autoantibody titers were examined in the combined cohorts. Of those who did not develop T1D (n=28,652), ICA512 and GAD65 titers were much more strongly correlated (r=0.31) than the titers of any other autoantibody pair (r ranged from 0.03 to 0.13). However, among those who developed T1D (n=383), whereas the ICA512-GAD65 correlation remained similar (r=0.30), the correlations of other autoantibody pairs tended to increase, especially when the pair included ICA titer (with ICA titer: r=0.39 for GAD65 titer; r=0.51 for ICA512 titer; r=0.34 for mIAA titer). In this large data set all correlations were significant (p<0.001 for all).

The association between the development of T1D and titer (log transformed) was examined among those who were positive for single autoantibodies in the combined cohorts. Due to the lack of T1D cases for those with mIAA positivity alone, the analysis was not performed for that autoantibody. GAD65 titer (T1D/Total=27/582) and ICA titer (29/472) were each predictive of T1D (p<0.01 for both, with and without age as a covariate). There was borderline significance (p=0.04 and p=0.07 with age added) for the ICA512 titer (6/113), but the number for that analysis was small.

CONCLUSIONS
The analyses presented above were designed specifically to discern the extent to which single positive autoantibodies predict the occurrence of T1D. They showed that among those in the Questionnaire cohort, ICA, ICA512, and GAD65, as single positive autoantibodies, were similarly predictive of T1D. Also, each of those autoantibodies appeared to add significant increments of risk when they were included as second autoantibodies in the Questionnaire cohort. However, T1D was not associated with mIAA as a single autoantibody, and the inclusion of mIAA as a second or a third autoantibody appeared to have little effect. The addition of single biochemical autoantibodies to ICA in the Trials cohort did not significantly increase T1D risk with age included as a covariate. This could be related to the selection criteria for that cohort and to small numbers.
The findings from this study are better understood by considering them in the context of the characteristics of each cohort. The vast majority of those in the Questionnaire cohort were ICA negative at initial screening, whereas those in the Trials cohort were mostly ICA positive at initial screening, and eventually all became positive prior to randomization. Moreover, the Trials cohort was selected for additional characteristics, including metabolic impairment and IAA positivity. These factors, together with the higher prevalence of ICA than that of other autoantibodies in the Trials cohort, could explain the relatively stronger association of T1D with single positivity of ICA when the cohorts were combined.

The finding that autoantibody number predicts T1D is consistent with other studies (9,15). The lack of a mIAA effect was similar to a previous finding of little effect when IAA was included as a third autoantibody (21). However, there have been other studies that showed a higher risk associated with IAA positivity (15,22,23). Other autoantibodies, either accounted for or unaccounted for, could have explained these associations. ICA positive individuals who were also positive for IA2βA have been observed to be at high risk for T1D (11). The addition of ICA to other autoantibodies has been shown to substantially enhance the risk of T1D in first-degree relatives (24). An autoantibody to zinc transporter 8 has recently been found to be predictive of T1D in children (12).

The percentages of common positivity with at least one other autoantibody ranged from 22% for mIAA to 65% for ICA512. Also, the correlations of titers between autoantibody pairs differed, and the correlations varied according to the subsequent development of T1D. It is possible that associations among autoantibodies according to positivity and titer are a function of the stage of progression to T1D in the study population. If so, the serial follow-up of these associations could provide insight into both the prediction and pathogenesis of T1D.

Among those with single positivity of GAD65 and ICA, T1D was predicted by titer. Thus, titers can provide useful information, even within the positive range. In a previous report, among those with positive autoantibodies, the titers of IA-2A and IAA were both predictive of T1D (25).

Insulin autoantibodies have been the most difficult to measure in multiple DASP workshops. Although a cutoff is set at the 99th percentile for each of the autoantibodies, the strength of signal for insulin autoantibodies of patients with new onset T1D is much closer to the range of signal for normal controls compared to either GAD65 or ICA512 autoantibodies. Thus, it is possible that a sizable proportion of mIAA positive values represent false positive or low affinity insulin autoantibodies associated with lower risk (26). Since confirmation and persistence was not determined in the current study, these factors will be important to evaluate in future studies.

The findings in this study might not be fully generalizable, since they were derived from relatives of patients with T1D. As discussed above, the Trials cohort was selected on the basis of certain criteria. In addition, the composition of the Questionnaire cohort could have been influenced by the willingness to respond to the questionnaire, and even to some extent by the absence of the qualifying criteria for entry into the trials. As discussed above, the increased occurrence of T1D in the Trials cohort is probably attributable to selection factors for trial entry and to OGTT surveillance.

Our findings show that although autoantibody number is a predictor of T1D, the particular type and titer of an autoantibody can influence prediction. Moreover, it is evident that the frequencies and the
associations of autoantibodies with each other can vary to a great extent. These factors must be carefully considered in planning prevention trials for T1D.
REFERENCES
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Table 1. Prevalence of Positive Autoantibodies at Initial Screening

<table>
<thead>
<tr>
<th></th>
<th>Questionnaire Cohort (n=28,507)</th>
<th>Trials Cohort (n=528)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (%)</td>
<td>N (%)</td>
</tr>
<tr>
<td>ICA</td>
<td>674 2.4</td>
<td>440 83.3</td>
</tr>
<tr>
<td>GAD65</td>
<td>907 3.2</td>
<td>363 68.8</td>
</tr>
<tr>
<td>ICA512</td>
<td>315 1.1</td>
<td>258 48.9</td>
</tr>
<tr>
<td>mIAA</td>
<td>525 1.8</td>
<td>136 25.8</td>
</tr>
</tbody>
</table>

Table 2. Associations* of T1D Occurrence with the Presence of Single Autoantibodies in the Questionnaire Cohort at Initial Screening

<table>
<thead>
<tr>
<th></th>
<th>T1D/Total</th>
<th>Percent</th>
<th>Hazard Ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAD65</td>
<td>25/568</td>
<td>4.4</td>
<td>27.6</td>
<td>16.8-45.4*</td>
</tr>
<tr>
<td>ICA512</td>
<td>5/110</td>
<td>4.6</td>
<td>29.5</td>
<td>11.6-74.7*</td>
</tr>
<tr>
<td>mIAA</td>
<td>0/407</td>
<td>0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>ICA</td>
<td>16/407</td>
<td>3.9</td>
<td>27.5</td>
<td>15.4-49.0*</td>
</tr>
</tbody>
</table>

* Reference Group: Negative for all autoantibodies (T1DTotal=41/26,651)

+ p<0.001

FIGURE LEGENDS

Figure 1. The curves indicate the occurrence of T1D over follow-up according to the number of autoantibodies present at the initial screening in the Questionnaire cohort, the Trials Cohort and in the cohorts combined. In all three panels there were significant trends among the groups of an increasing occurrence of T1D with increasing autoantibody number. The numbers (1-4) indicate the number of autoantibodies. (The fraction in parentheses indicates the number who developed T1D among the number in the group at baseline.)

Figure 2. The curves indicate the occurrence of T1D in the Questionnaire cohort over follow-up for the presence of one autoantibody and for the additional presence of a second autoantibody at the initial screening. Thus, in each panel the groups that included a specific second positive autoantibody was compared with the group that had one positive autoantibody from any of the others. The panels show that when ICA, GAD65, and ICA512 each were present as second autoantibodies, there were significant and similar increases in the occurrence of T1D; however, there was no increase when mIAA was present as a second autoantibody. The number shown for each curve (1,2) indicates the number of autoantibodies. Proportions of those who developed T1D are shown for each curve. (The fraction in parentheses indicates the number who developed T1D among the number in the group at baseline.)
Figure 1

**Questionnaire**

- Proportion without TID
- Years: 0, 2, 4, 6, 8, 10
- TID/Total: 0=(41/26,651), 1=(46/1,492), 2=(32/199), 3=(52/129), 4=(18/36)
- p=0.001

**Trials**

- Proportion without TID
- Years: 0, 2, 4, 6, 8, 10, 12
- TID/Total: 0=(14/56), 1=(16/85), 2=(48/125), 3=(75/186), 4=(41/76)
- p=0.001

**Both**

- Proportion without TID
- Years: 0, 2, 4, 6, 8, 10, 12
- TID/Total: 0=(55/26,707), 1=(62/1,577), 2=(80/324), 3=(127/315), 4=(59/112)
- p=0.001
Figure 2

Addition of ICA

Addition of GAD65

Addition of ICA512

Addition of mIAA