Zinc Transporter-8 Autoantibodies Improve Prediction of Type 1 Diabetes in Relatives Positive for the Standard Biochemical Autoantibodies

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For the Type 1 Diabetes TrialNet Study Group*8

OBJECTIVE—We assessed diabetes risk associated with zinc transporter-8 antibodies (ZnT8A), islet cell antibodies (ICA), and HLA type and age in relatives of people with type 1 diabetes with the standard biochemical autoantibodies (BAA) to insulin (IAA), GAD65 (GAD65A), and/or insulinoma-associated protein 2 antigen (IA-2A).

RESEARCH DESIGN AND METHODS—For this analysis, 2,256 relatives positive for at least one BAA, of whom 142 developed diabetes, were tested for ZnT8A, ICA, and HLA genotype followed by biannual oral glucose tolerance tests. ZnT8A were also tested in 911 randomly chosen antibody-negative relatives.

RESULTS—ZnT8A were associated with the other BAA (548 of 2,256 [24.3%] BAA+ vs. 8 of 911 [0.8%] BAA+, P < 0.001) and BAA number (177 of 1,683 [10.5%] single-, 221 of 384 [57.6%] double-, and 150 of 189 [79.4%] triple-BAA positivity, P < 0.001). The 4-year diabetes risk was higher in single BAA+ relatives with ZnT8A than ZnT8A− relatives (31 vs. 7%, P < 0.001). In multivariable analysis, age ≤20 years (hazard ratio 2.13, P = 0.03), IA-2A (2.15, P = 0.005), IAA (1.73, P = 0.01), ICA (2.37, P = 0.002), and ZnT8A (1.87, P = 0.03) independently predicted diabetes, whereas HLA type (high and moderate vs. low risk) and GAD65A did not (P=0.81 and 0.86, respectively).

CONCLUSIONS—In relatives with one standard BAA, ZnT8A identified a subset at higher diabetes risk. ZnT8A predicted diabetes independently of ICA, the standard BAA, age, and HLA type. ZnT8A should be included in type 1 diabetes prediction and prevention studies.

Type 1 diabetes is usually preceded by a subclinical prodrome marked by islet cell antibodies (ICA) and biochemical autoantibodies (BAA) to insulin (IAA), GAD65 (GAD65A), and the insulinoma-associated protein 2 antigen (IA-2A/ICA512A) (1). The predictive validity of the autoantibodies for diabetes in relatives of people with type 1 diabetes has made autoantibody positivity an entry criterion for type 1 diabetes secondary prevention trials (2–5) and a surrogate outcome in primary prevention trials (6). Autoantibodies to the islet antigen zinc transporter-8 (ZnT8A) recently were found to predict type 1 diabetes (7–9). However, the relationship between diabetes risk and ZnT8A in combination with other risk markers, including ICA, the standard BAA, HLA genotype, and age, remains unclear.

We therefore measured ZnT8A in a large cohort of relatives being followed in the TrialNet Natural History Study of Type 1 Diabetes (NHS). We hypothesized that ZnT8A positivity would increase diabetes risk in relatives positive for a single BAA—a group that accounts for most autoantibody-positive relatives but whose members are at much lower risk compared with relatives with two or more autoantibodies (10). We also assessed whether ZnT8A increased diabetes risk independently of ICA, the BAA, HLA class II genotype, and age.

RESEARCH DESIGN AND METHODS—All participants were enrolled in the TrialNet NHS between 2004 and 2008. The NHS is an ongoing prospective cohort study with the aims to find subjects for type 1 diabetes prevention trials and to assess the natural history of pre-type 1 diabetes according to established and new diabetes risk markers (11). Nondiabetic first-degree (age 1–45 years) and second/third-degree (age 1–20 years) relatives of people with type 1 diabetes were screened for IAA, GAD65A, and IA-2A. Subjects with a single BAA were invited to return for a second autoantibody test, and both samples were tested for ICA as well. Subjects positive for more than two BAA on the first test, or more than two autoantibodies, including ICA, on two separate screening tests, were offered follow-up HLA typing and biannual oral glucose tolerance tests (11). For this analysis, 2,256 relatives positive for greater than one BAA on their first screening test were identified, and their baseline screening sample was tested for ZnT8A. To mask laboratory personnel,
and to estimate the prevalence of ZnT8A among relatives negative for the BAA, ZnT8A were also tested in baseline samples from 911 randomly chosen BAA- relatives.

**Laboratory methods**

HLA-DQ polymorphisms were determined by allele-specific oligonucleotide genotyping (12). The haplotypes of interest were DQA1*0501-DQB1*0201 (DQ2), DQA1*0301-DQB1*0302 (DQ8), and DQA1*01-DQB1*0602 (DQ6). ICA, GAD65A, IA-2A, and micro IAA were measured in TrialNet Core Laboratories (University of Florida, Gainesville [ICA]; Barbara Davis Center for Childhood Diabetes [BAA]) using previously described methods and cut points to define positivity (13,14). In the 1998 Combinatorial Islet Antibody Workshop, the sensitivity and specificity for ICA was, respectively, 81 and 96% (15). In the 2009 Diabetes Autoantibody Standardization Program (DASP) workshop, the respective sensitivities and specificities were 66 and 99% for GAD65A and 62 and 99% for IA-2A. In the 2007 DASP workshop, the sensitivity and specificity for IAA was, respectively, 66 and 99%.

For ZnT8A, the dimer protein ZnT8WR was synthesized via in vitro transcription/translation using the TNT kit (Promega) and labeled with 35-S methionine (PerkinElmer) (7). Serum (2 μL) was incubated with 50 μL labeled ZnT8WR (20,000 cpm) and precipitated with protein A Sepharose (GE Healthcare). The assay was performed in a 96-well filtration plate (Fisher Scientific), and radioactivity was determined on a Topcount 96-well plate β-counter (PerkinElmer). The antibody levels were expressed as an index \(\frac{\text{cpm of sample} - \text{cpm of negative control}}{\text{cpm of positive control} - \text{cpm of negative control}}\). The interassay coefficient of variation was 10.2% \((n=20)\), and the upper limit of normal controls (0.020) was established as the 99th percentile of 100 healthy control subjects. In the 2010 DASP workshop, the assay achieved 64% sensitivity with 100% specificity.

**Sample size and statistical analysis**

Before the study, we determined that there would be 80% power (5% significance level) to detect hazard ratios for diabetes as small as 2.0 between ZnT8A+ and ZnT8A- relatives also positive for one standard BAA. The power projections were based on ascertaining at least 1,900 BAA+ relatives and varying assumptions across a range of plausible rates for ZnT8A prevalence in BAA+ relatives (5–10%) and 5-year diabetes risks among single BAA+ relatives who were also ZnT8A- (5–10%). The main outcome was diabetes by 2009 American Diabetes Association criteria (16). Categorical variables between groups were compared by the \(\chi^2\) test. Survival analysis for diabetes onset was limited to autoantibody-positive relatives and used the Kaplan-Meier method. The log-rank test was used to compare cumulative incidence of diabetes between groups. Time to onset of diabetes by individual and combined risk markers, including age at the first autoantibody test (≤20 or >20 years), the specific autoantibody (positive or negative), and HLA type (high risk: DQ2/DQ8; moderate risk: DQ2/DQ8, DQ8/DQ8, or DQ8/X; and low risk: DQ6/X, XX, or DQ2/X), was assessed by Cox proportional hazards regression model. Two multivariable regressions were done using backward stepwise selection (significance level to stay = 0.05). The first regression included participants who contributed samples for HLA typing \((n=723)\) on a follow-up visit. The second regression used a larger \((n=1,767)\) cohort of participants with results available at the first screening test (age and autoantibodies but not HLA type). The statistical analyses used SAS software, \(P\) values were not adjusted for multiple comparisons, and a \(P\) value (two-tailed) of ≤0.05 was considered significant.

**RESULTS**

Of 2,256 relatives positive for at least one BAA on the first screening test, 486 (22%) did not return for follow-up. There were differences between this group and relatives who provided follow-up. There were differences between this group and relatives who provided follow-up, \(P=0.004\) and multiple \((P=0.02)\) but not in sex \((P=0.03)\). Among the 1,770 relatives who were followed up, 142 developed diabetes after a mean of 1.3 years \((range 0.02–4.9 years)\).

Table 1 shows the prevalence of autoantibodies on the first screening test among BAA+ relatives. ZnT8A were found in 548 of 2,256 (24.3%) relatives positive for at least one BAA but were much less prevalent in BAA- relatives \((8 of 911 [0.9%], P<0.001)\) vs. BAA+ relatives. ZnT8A were strongly associated with the number of positive BAA, being present in 177 of 1,683 (10.5%) single BAA+, 221 of 384 (57.6%) double BAA+, and 150 of 189 (79.4%) triple BAA+ relatives \((P<0.001)\). ZnT8A were also associated with autoantibody type among single BAA+ relatives:

<table>
<thead>
<tr>
<th>Autoantibody</th>
<th>BAA+ relatives*</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>2,256</td>
</tr>
<tr>
<td>GAD65A</td>
<td>1,669 (74.0)</td>
</tr>
<tr>
<td>IAA</td>
<td>752 (33.3)</td>
</tr>
<tr>
<td>IA-2A</td>
<td>597 (26.5)</td>
</tr>
<tr>
<td>ICA</td>
<td>574 (25.4)</td>
</tr>
<tr>
<td>ZnT8A</td>
<td>548 (24.3)</td>
</tr>
<tr>
<td>One BAA</td>
<td>1,683 (74.6)</td>
</tr>
<tr>
<td>Two BAA</td>
<td>384 (17.0)</td>
</tr>
<tr>
<td>Three BAA</td>
<td>189 (8.4)</td>
</tr>
<tr>
<td>GAD65A only</td>
<td>1,140 (50.5)</td>
</tr>
<tr>
<td>IAA only</td>
<td>379 (16.8)</td>
</tr>
<tr>
<td>IA-2A only</td>
<td>164 (7.3)</td>
</tr>
<tr>
<td>One BAA and ZnT8A</td>
<td>177/1,683 (10.5)</td>
</tr>
<tr>
<td>GAD65A/ZnT8A</td>
<td>109/177 (62)</td>
</tr>
<tr>
<td>IA-2A/ZnT8A</td>
<td>49/177 (28)</td>
</tr>
<tr>
<td>IAA/ZnT8A</td>
<td>19/177 (11)</td>
</tr>
<tr>
<td>Two BAA and ZnT8A</td>
<td>221/384 (57.6)</td>
</tr>
<tr>
<td>Three BAA and ZnT8A</td>
<td>150/189 (79.4)</td>
</tr>
</tbody>
</table>

Data are n (%) where \(N=2,256\) or n/n% with \(N\) as indicated. *BAA* refers to positivity for at least one of GAD65A, IAA, and IA-2A.
BAA, subdivided by ZnT8A. The risk for diabetes was significantly higher ($P = 0.0001$) among multiple BAA+ relatives who were also ZnT8A+ compared with those who were ZnT8A−. The increased risk in ZnT8A+ relatives was concentrated in the subgroup with two BAA ($P = 0.0013$) (Supplementary Fig. 2). In relatives positive for all three standard BAA, diabetes risk was higher if they were also positive for ZnT8A, but this difference was not statistically significant compared with ZnT8A− relatives ($P = 0.067$) (Supplementary Fig. 3).

The risk for diabetes increased incrementally according to the number of positive tests for the standard BAA, ZnT8A, and ICA. Thus, the 3-year cumulative diabetes incidences (95% confidence limits) in relatives positive for two, three, four, and five autoantibodies were, respectively, 10 (6–15), 28 (21–36), 35 (18–62), and 52% (40–65).

The added impact of ICA positivity on diabetes risks in relatives positive for one or more of the standard BAA and ZnT8A is shown in Fig. 3 and Supplementary Figs. 4–6. The point estimates for diabetes risks by the 2nd year of follow-up were higher among ICA+ compared with ICA− relatives irrespective of the number of other positive autoantibodies. The difference was statistically significant among ICA+ relatives with two other antibodies ($P < 0.0001$) (Fig. 3) and with one of the standard BAA ($P = 0.05$) (Supplementary Fig. 4) but not in relatives positive for three ($P = 0.07$) and four ($P = 0.56$) other autoantibodies (Supplementary Figs. 5 and 6).

In the proportional hazards regression that included HLA type ($n = 723$ subjects, $n = 95$ diabetic case subjects), age ≥20 years (hazard ratio 2.13, $P = 0.03$) and positive tests for IA-2A (2.15, $P = 0.005$), IAA (1.73, $P = 0.01$), ICA (2.37, $P = 0.002$), and ZnT8A (1.87, $P = 0.03$) were independently predictive of diabetes, whereas HLA type (high vs. low; moderate vs. low) and GAD65A positivity were not (adjusted $P$ values = 0.81 and 0.86, respectively). In the model limited to age and autoantibodies ($n = 1,767$ subjects, $n = 142$ diabetic case subjects), age ≥20 years (1.77, $P = 0.03$) and positivity for IA-2A (2.17, $P = 0.004$), IAA (1.46, $P = 0.03$), ICA (2.33, $P < 0.0001$), and ZnT8A (2.65, $P < 0.0001$) independently predicted diabetes, but GAD65A were again not retained (adjusted $P$ value = 0.55). These findings were similar in proportional hazards regressions using forward stepwise selection and that included sex as an additional variable.

**CONCLUSIONS**—In relatives of people with type 1 diabetes positive for one or more of the standard diabetes-associated BAA (IAA, GAD65A, or IA-2A), we found that ZnT8A testing added useful information about diabetes risk. We confirmed
were BAA + (we had larger numbers of relatives who
ous studies showing an association between
Compared with previous studies (7
ZnT8A, the standard BAA, and ICA.
characterized cohort of relatives tested for
date. Our results con
by the shaded area. Diabetes risk was higher among ICA+ relatives (P < 0.0001). AB, antibody.

Figure 3 — The cumulative incidence of diabetes in relatives positive for two BAA (any two of GAD65A, IAA, IA-2A, and ZnT8A) with or without ICA. The 95% confidence limits are indicated by the shaded area. Diabetes risk was higher among ICA+ relatives (P < 0.0001). AB, antibody.

our a priori hypothesis that ZnT8A pos-
tivity increased risk in relatives positive for a single standard BAA. We also found that
ZnT8A positivity increased risk in multiple BAA+ relatives and that ZnT8A remained predictive of diabetes after ad-
justment for age, HLA type, and positivity for the standard BAA and ICA. As well, we
found that ICA contributed to risk be-
yond the autoantibodies to the four bio-
chemically defined antigens identified to
date. Our results confirm and extend previ-
ous studies showing an association between
ZnT8A and subsequent diabetes (7–9).
Our study’s main strength was the
prospective observation of a large, well-
characterized cohort of relatives tested for
ZnT8A, the standard BAA, and ICA.
Compared with previous studies (7–9),
we had larger numbers of relatives who
were BAA+ (N = 2,256), ZnT8A+ (n = 548), and who developed diabetes (n = 142). This increased the power to detect
associations between ZnT8A and diabetes
risk, including risk independent of other
markers in multivariable analyses. Other
strengths include use of validated autoan-
tibody assays and because participants
entering TrialNet prevention trials must
do so through the NHS, assessment of a
cohort that is similar in age and genetic
risk to those participating in current and
future TrialNet prevention studies.

Our findings have implications for
type 1 diabetes prediction and prevention
studies. Foremost, the independent and
consistent relationship between ZnT8A
and diabetes risk seen not only here but
also in three other studies (7–9) strongly
supports ZnT8A testing in prediction and
prevention studies. For example, testing
for ZnT8A in relatives positive for a single
standard BAA found a subgroup at much
higher diabetes risk (31 vs. 7% per
4 years). Although only 8% of single BAA+
relatives were also ZnT8A+, the high preva-
ience of single BAA positivity (~75%)
means that an appreciable number of
higher risk relatives with more than two
autoantibodies to biochemically defined
antigens, including ZnT8, will be missed
if ZnT8A are not measured. ZnT8A test-
ing also refined risk estimation in multi-
ple autoantibody positive (more than two
standard BAA) relatives by identifying a
ZnT8A+ group at higher risk. While other
studies find a direct association between
the number of positive autoantibodies and
diabetes risk (1,10), ours is the first to
show that ZnT8A incrementally add risk
over the standard BAA and ICA. Given
these findings, measurement of ZnT8A
in relatives positive for at least one stan-
ard BAA (“secondary” testing) has been
incorporated into TrialNet’s screening
protocol.

Our results also have potential path-
ogenic implications. The sharp rise in
ZnT8A prevalence as the number of pos-
itive standard BAA increased, with corre-
spondingly higher diabetes risks, suggests
that ZnT8A expression is a nonspecific
and later by-product of underlying pa-
thology rather than a consequence of
unique factors that target ZnT8. As De
Grijs et al. (9) noted, because ZnT8 is
located within β-cell secretory granules,
ZnT8A expression may not occur until
there is enough β-cell damage to make
ZnT8 immunologically visible. However,
Achenbach et al. (8) found relationships
between diabetes risk and genotypes of
the ZnT8-encoding gene SLC30A8 in
ZnT8A+ children, indicating that in some
cases, there may be interactions between
specific genetic factors, risk, and, ZnT8A
expression. The persistent association
between ICA positivity and diabetes risk af-
er adjustment for positive tests for BAA
and ZnT8 implies the existence of other
as yet unidentified autoantibodies to spe-
cific antigens. This finding also supports
continued use of the relatively nonspec-
cific, labor-intensive ICA determination
as a secondary autoantibody test in pre-
diction and prevention trials.

The failure of GAD65A to indepen-
dently predict diabetes in the multivari-
able analyses was unexpected. This may
not reflect pathogenesis but, rather, prop-
erties specific to our cohort (including
the high prevalence of GAD65A [74%],
the fact that all participants followed for
diabetes were positive for at least one
autoantibody, and the tendency for
GAD65A to occur in older relatives at
lower diabetes risk) that reduced the
power to detect an independent associa-
tion between GAD65A and diabetes in
the multivariable models. As well, other
studies test ZnT8A and the standard
BAA, including GADA, in both older
(17) and younger (18) patients with estab-
lished diabetes and find that GADA added
information about clinical phenotypes.
It may therefore be premature to dis-
count GAD65A testing in type 1 diabe-
tes prediction.

Our study has limitations. Our follow-
up was shorter compared with other
studies assessing ZnT8A (mean = 1.3
years vs. 10.8 years [8] and 5.7 years
[9]), and we did not measure IA-2B au-
toantibodies, which have been found to
predict diabetes (9,19). Although our re-
sults might suggest that ZnT8A occur later
in pre–type 1 diabetes compared with
the other autoantibodies, this is based on
prevalence data. Serial measurements of ZnT8A and the other autoantibodies in autoantibody-negative cohorts are needed to be sure about temporality. Related to this, the low prevalence of isolated ZnT8A positivity we saw (0.9%) in BAA–relatives could suggest that adding ZnT8A to the standard BAA on the first screening test will be of less value where “value” is based on identifying a significant number of additional relatives at higher risk. However, we did not assess progression to multiple autoantibody positivity or diabetes in relatives with only ZnT8A and, therefore, cannot rule out a role for including ZnT8A as a primary screening test. Finally, we did not add metabolic predictors, including abnormal oral glucose tolerance (2), the Diabetes Prevention Trial Risk Score (20), insulin sensitivity (21), or A1C levels (22), to the Cox analyses because the number of diabetic case subjects \( n = 94 \) was insufficient relative to the number of independent variables that would be tested in more comprehensive models (23).

In conclusion, ZnT8A strongly predicted diabetes in relatives of people with type 1 diabetes. This relationship was independent of ICA, the standard BAA, age, and HLA type. Among relatives positive for a single standard BAA or who were positive for more than two BAA, ZnT8A testing identified subsets at higher diabetes risks and should be included in type 1 diabetes prediction and prevention studies.

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L.Y., D.C.B., C.J.G., J.P.K., and G.S.E. researched data, contributed to discussion, and reviewed the manuscript. C.A.B., J.C.H., and J.M.W. researched data and reviewed the manuscript. P.J.B. researched data, contributed to discussion, and edited the manuscript. J.M.S. contributed to discussion and reviewed the manuscript. J.S.S. researched data and reviewed the manuscript. L.Y., D.C.B., C.A.B., J.C.H., J.M.W., C.J.G., P.J.B., J.P.K., J.M.S., J.S.S., G.S.E., and J.M.L. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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