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

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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.

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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 at least one BAA on their first

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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 {[(cpm of sample) – (cpm of negative control)]/[(cpm of positive control) – (cpm of negative control)]}. The interassay coefficient of variation is 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 χ^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/DQ2, DQ8/DQ8, or DQ8/X; and low risk: DQ6/X, X/X, 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 in age (mean = 22.4 vs. 20.4 years, respectively, P = 0.004) and multiple (greater than two) BAA positivity (21 vs. 27%, P = 0.02) but not in sex (female 57 vs. 59%, P = 0.43). 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: ZnT8A were detected in 19 of 177 relatives (11%) with IAA, 109 of 177 (62%) with GAD65A, and 49 of 177 (28%) with IA-2A ($P < 0.001$). ZnT8A were more common in younger participants (454 of 1,316 [34.5%] aged <20 years vs. 94 of 940 [10.0%] relatives aged >20 years, $P < 0.0001$).

Table 1—*Prevalence of autoantibodies at screening*

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

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.

Samples for HLA typing were obtained in 723 BAA^+ relatives. The prevalence of ZnT8A was strongly associated with high and moderate HLA risk genotypes compared with low risk genotypes (168 of 424 [39.6%] vs. 69 of 299 [23.1%], respectively, $P < 0.0001$). Among relatives with high and moderate risk genotypes, the respective $ZnT8A^+$ rates were 60 of 126 (47.6%) and 108 of 298 (36.2%) (P = 0.03).

Figure 1 shows the cumulative incidence of diabetes in single standard BAA^+ relatives subdivided by ZnT8A. ZnT8A were strongly associated with onset of diabetes, with an estimated 4-year risk (95% confidence limit) of 31% (19–46) compared with 7% (4–11) ($P < 0.001$) among $ZnT8A^-$ relatives. In relatives positive for ZnT8A and one other BAA, the 3-year cumulative diabetes incidence varied with the antibodies detected

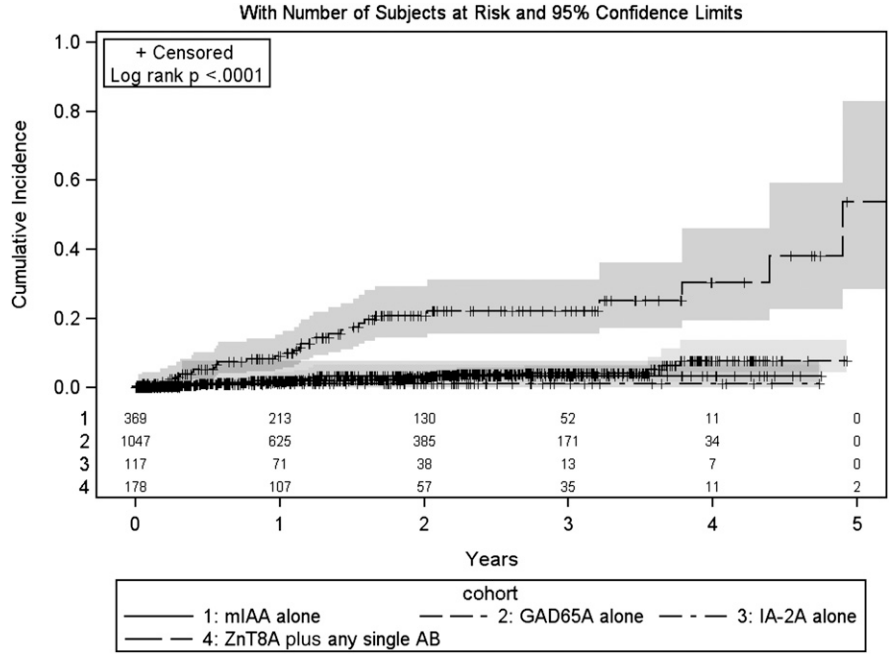

Figure 1—*Cumulative incidences of diabetes in relatives positive for one standard BAA (IAA, GAD65A, or IA-2A) with or without ZnT8A. ICA$^+$ relatives are excluded. The 95% confidence limits are indicated by the shaded areas. Diabetes risk was higher among ZnT8A$^+$ relatives (*P < *0.0001). mIAA, micro IAA; AB, antibody.*

(GAD65A, 9%; IAA, 41%; and IA-2A, 45%; $P = 0.0002$) (Supplementary Fig. 1).

Figure 2 shows diabetes risks in relatives positive for at least two standard 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).

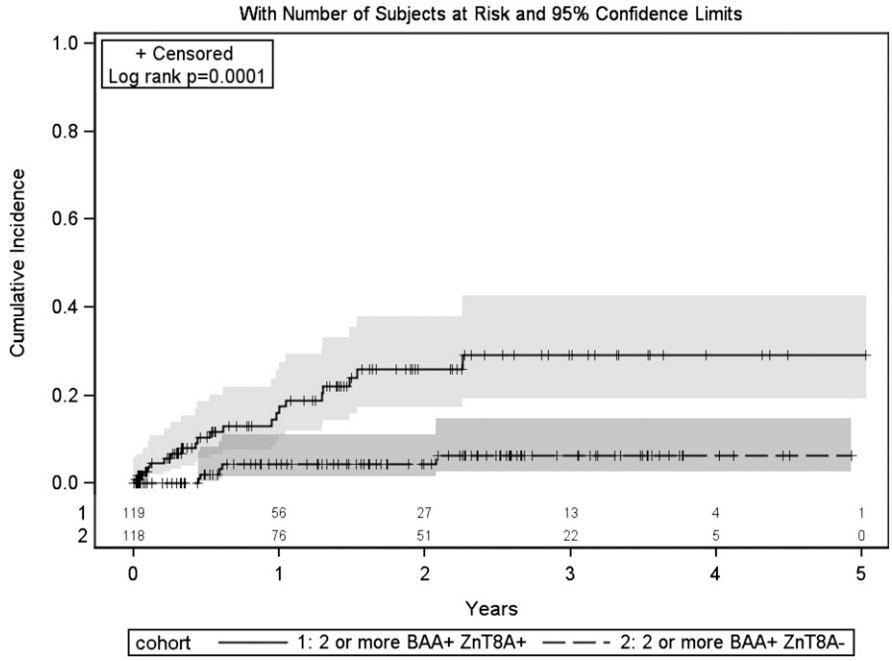

Figure 2—*Cumulative incidences of diabetes in multiple autoantibody (at least two standard BAA) positive relatives with or without ZnT8A. ICA$^+$ relatives are excluded. The 95% confidence limits are indicated by the shaded areas. Diabetes risk was higher among ZnT8A$^+$ relatives (*P = *0.0001).*

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

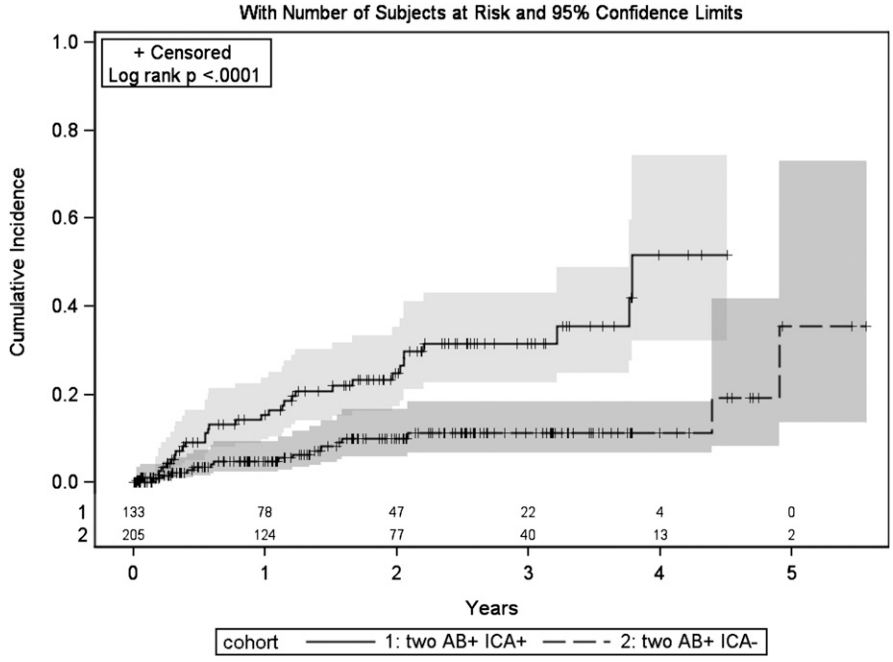

Figure 3—*The cumulative incidence of diabetes in relatives positive for two BAA (any two of GAD65A, IAA, IA-2A, and ZnT8A) with or without ICA. The 95% confidence limits are indicated by the shaded area. Diabetes risk was higher among ICA$^+$ relatives ($P < 0.0001$). AB, antibody.*

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 our a priori hypothesis that ZnT8A positivity 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 adjustment for age, HLA type, and positivity for the standard BAA and ICA. As well, we found that ICA contributed to risk beyond the autoantibodies to the four biochemically defined antigens identified to date. Our results confirm and extend previous 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 autoantibody 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 prevalence 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 testing also refined risk estimation in multiple 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 standard BAA ("secondary" testing) has been incorporated into TrialNet's screening protocol.

Our results also have potential pathogenic implications. The sharp rise in ZnT8A prevalence as the number of positive standard BAA increased, with correspondingly higher diabetes risks, suggests that ZnT8A expression is a nonspecific and later by-product of underlying pathology rather than a consequence of unique factors that target ZnT8. As De Grijse 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 after adjustment for positive tests for BAA and ZnT8A implies the existence of other as yet unidentified autoantibodies to specific antigens. This finding also supports continued use of the relatively nonspecific, labor-intensive ICA determination as a secondary autoantibody test in prediction and prevention trials.

The failure of GAD65A to independently predict diabetes in the multivariable analyses was unexpected. This may not reflect pathogenesis but, rather, properties 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 association 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 established diabetes and find that GADA added information about clinical phenotypes. It may therefore be premature to discount GAD65A testing in type 1 diabetes 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-2β autoantibodies, which have been found to

predict diabetes (9,19). Although our results 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. J.L.M. researched data and wrote 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.L.M. 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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