Positivity for Zinc Transporter 8 Autoantibodies at Diagnosis Is Subsequently Associated With Reduced β-Cell Function and Higher Exogenous Insulin Requirement in Children and Adolescents With Type 1 Diabetes

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OBJECTIVE
This study assessed the relationship between autoantibodies against zinc transporter 8 (ZnT8A) and disease characteristics at diagnosis of type 1 diabetes and during the first 2 years.

RESEARCH DESIGN AND METHODS
Children, younger than 15 years of age (n = 723) who were newly diagnosed with diabetes, were analyzed for ZnT8A, other diabetes-associated autoantibodies, HLA DR-DQ alleles, and metabolic status, which was monitored by pH, plasma glucose, and occurrence of ketoacidosis at diagnosis and through follow-up of C-peptide concentrations, exogenous insulin dose, and glycosylated hemoglobin for 2 years after the diagnosis.

RESULTS
ZnT8A positivity was detected in 530 children (73%). Positivity for ZnT8A was associated with older age (median 8.9 vs. 8.2 years, \( P = 0.002 \)) and more frequent ketoacidosis (24% vs. 15%, \( P = 0.013 \)). Children carrying the HLA DR3 allele were less often ZnT8A positive (66% vs. 77%, \( P = 0.002 \)) than others. ZnT8A-positive children had lower serum C-peptide concentrations (\( P = 0.008 \)) and higher insulin doses (\( P = 0.012 \)) over time than their ZnT8A-negative peers.

CONCLUSIONS
Positivity for ZnT8A at diagnosis seems to reflect a more aggressive disease process before and after diagnosis.

The clinical diagnosis of type 1 diabetes is preceded by an asymptomatic preclinical phase, during which autoantibodies against intracellular antigens of the β-cells appear in the circulation (1). Zinc transporter 8 (ZnT8) is the most recently discovered diabetes-associated autoantigen (2). The aim of this study was to assess the relationship between ZnT8A on the one hand and demographic characteristics, other diabetes-associated autoantibodies, HLA risk markers, the degree of

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metabolic decompensation at diagnosis, and the natural course of the disease during the first 2 years after diagnosis on the other hand.

**RESEARCH DESIGN AND METHODS**

**Subjects**
The population-based, nationwide Childhood Diabetes in Finland (DiMe) study was conducted from 1986 to 1989. All patients younger than 15 years who were diagnosed with type 1 diabetes according to the World Health Organization criteria were invited to participate. The study involved 801 participants; serum samples were initially available from 758 children. The current study population comprised 723 children (55.4% male) because no serum was any longer available from 35 children (55.4% male) because no serum was any longer available from 35 children. These 723 patients represent the index cases in the DiMe study, described in detail elsewhere (3). The ethical committees of all participating hospitals approved the study protocol, and the parents gave written informed consent to their child’s participation. Serum samples were stored at −70°C.

**Autoantibody Assays**
Serum ZnT8A levels were analyzed by a radiobinding assay as described earlier (4,5). Islet cell antibody (ICA) was detected with indirect immunofluorescence, whereas GAD antibody (GADA), IA-2A, and insulin autoantibody (IAA) were quantified with specific radiobinding assays (6). We used a cutoff limit for ICA positivity of 2.5 JDFU. Antibody levels for ZnT8A, GADA, IA-2A, and IAA were expressed in relative units (RU). The cutoff limits corresponding to the 99th percentile in 374 nondiabetic children are 0.61 RU for ZnT8A, 3.48 RU for IA-2A, and 0.43 RU for IAA. The disease sensitivity and specificity of the ZnT8A assay were 60% and 100%, respectively, according to the 2010 Diabetes Autoantibody Standardization Program.

**HLA Typing**
HLA typing of the main predisposing DQA1-DQB1 genotypes and DRB1*04 subtypes was performed with a PCR-based oligonucleotide hybridization and time-resolved fluorometry (7). The DR3-DQA1*05-DQB1*02 haplotype has been shortened to DR3 and HLA-DRB1*04-DQB1*0302 to DR4. HLA typing data were available for 682 patients.

**Markers of Metabolic Status**
Metabolic parameters included pH and plasma glucose at diagnosis, analyzed in the local laboratories. Diabetic ketoacidosis was defined as blood pH ≤7.30.

Random serum C-peptide concentrations, glycosylated hemoglobin (Ghb), and exogenous insulin dose were monitored for 2 years after diagnosis. C-peptide concentrations were measured with a radioimmunoassay (8), using antiserum K6 (Novo Research Institute, Bagsvaerd, Denmark). The intraassay coefficient of variation was 1.8%, and the interassay coefficient of variation was 10%. We have previously shown that random serum C-peptide levels correlate strongly with other standardized C-peptide analyses (9). Standard methods for blood GHb analyses were used in the various hospitals. To compare the results, data were expressed as SD above mean for subjects without diabetes in each laboratory (10).

**Statistical Analysis**
The data were statistically evaluated using cross-tabulation, the Kruskal-Wallis test, and the Mann-Whitney U test. Mixed between- and within-subjects ANOVA was used to explore the effect of ZnT8A positivity on serum C-peptide concentrations, Ghb levels, and the exogenous insulin dose during the 2-year follow-up period. Serum C-peptide concentrations were not normally distributed and were log transformed. Statistical analyses were performed with SPSS 21.0 software (IBM Corp., Armonk, NY). A P value <0.05 was considered significant.

**RESULTS**

**Frequencies and Levels of ZnT8A**
Of 723 children tested for ZnT8A, 530 (73.3%) were positive (median 4.13 RU, range 0.61–733.8 RU). The ZnT8A-positive children were generally older (median age 8.85 vs. 8.23 years, P = 0.002).

**Combinations and Associations Between Autoantibodies**
ZnT8A positivity concurred consistently with ICA and/or IA-2A positivity but less often with GADA- or IAA-positivity. These proportions varied according to age (Supplementary Table 1). When analyzed for the four classical antibodies—ICA, IAA, GADA, and IA-2A—13 children (1.8%) had no detectable antibodies. When ZnT8A was added, this number decreased by 31% (n = 9). The most sensitive (97.9%) combination conferred by three autoantibody assays was the analysis of ZnT8A, GADA, and IA-2A, leaving only 2.1% of the subjects undetected.

**The Relationship Between ZnT8A and HLA Genotypes**
Among the 226 children carrying the HLA DR3 allele, only 65.9% tested positive for ZnT8A, whereas 76.7% of the DR3-negative subjects were ZnT8A positive (P < 0.001). Of the HLA-associated genotypes, the highest frequency (79.9%) and median ZnT8A titer levels (3.0 RU, P = 0.009) were seen among those carrying the non-DR3/DR4 genotype.

**Metabolic State at Diagnosis**
At diagnosis, children who were positive for ZnT8A had lower blood pH (mean 7.33 vs. 7.36, P = 0.002) and were more likely to have ketoacidosis than children who were negative for ZnT8A (23.9% vs. 15.0%, P = 0.013).

**Natural Course of Type 1 Diabetes**
At the time of diagnosis, ZnT8A-positive and -negative children had serum C-peptide concentrations of the same magnitude and a similar need of exogenous insulin. The serum C-peptide concentrations were, however, higher in the ZnT8A-negative patients during the follow-up period (P = 0.008). When the serum C-peptide concentrations were compared at specific times, these were lower in the ZnT8A-positive children during the second year (Fig. 1A). The daily insulin dose was significantly higher in ZnT8A-positive children during the follow-up (Fig. 1B, P = 0.012). There was no significant difference in Ghb over time (Fig. 1C).

**CONCLUSIONS**
The association between ZnT8A positivity, C-peptide concentrations, and insulin requirement over the first 2 years suggests that a strong initial humoral immune response against ZnT8 suppresses the recovery ability of the residual β-cell function after diagnosis. ZnT8 might be a regulator of β-cell function, the observed association between a polymorphism in the SLC30A8 gene and type 2 diabetes supporting such a role (11). Positivity for ZnT8A increases...
the risk of ketoacidosis at diagnosis, reflecting a more aggressive disease.

In this study we observed a strong inverse relationship between the HLA DR3 allele and ZnT8A, suggesting that the DR3-DQ2 haplotype protects against autoimmunity to ZnT8. This view is supported by a recent report on T-cell responses to ZnT8 in type 1 diabetes (12). Although our study indicates that the frequency of ZnT8A is reduced in DR3-positive individuals, high GADA levels have been reported to be associated with HLA DR3 and high IA-2A titers with DR4 (7). These three autoantibodies may accordingly complement each other in relation to HLA associations.

Prospective studies have shown that positivity for two or more diabetes-associated autoantibodies is a strong predictor of progression to type 1 diabetes, with ~70% of such individuals presenting...
with clinical disease during a 10-year follow-up (13). The current results suggest that the combination of GADA, ZnT8A, and IA-2A assays might be a feasible and cost-effective approach for the detection of β-cell autoimmunity because only 2.1% of the subjects in the current study would have remained undetected.

Positivity for ZnT8A at diagnosis was associated with reduced β-cell function, increased insulin requirement, and a higher frequency of diabetic ketoacidosis at diagnosis. Altogether, positivity for ZnT8A seems to reflect a more aggressive disease process both before and after diagnosis.

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