CLINICAL STUDY

Danish children born with glutamic acid decarboxylase-65 and islet antigen-2 autoantibodies at birth had an increased risk to develop type 1 diabetes

Stefanie Eising¹, Anita Nilsson², Bendix Carstensen¹, David M Hougaard³, Bent Nørgaard-Pedersen³, Jørn Nerup¹, Åke Lernmark² and Flemming Pociot^{1,2,4}

¹Hagedorn Research Institute and Steno Diabetes Center, DK-2828 Gentofte, Denmark, ²Department of Clinical Sciences, Skåne University Hospital SUS, Lund University/CRC, SE-20502 Malmö, Sweden, ³Department of Clinical Biochemistry, Statens Serum Institut, DK-2300 Copenhagen, Denmark and ⁴Glostrup Research Institute, Glostrup University Hospital, Ndr. Ringvej 69, DK-2600 Glostrup, Denmark

(Correspondence should be addressed to F Pociot at Glostrup Research Institute, Glostrup University Hospital; Email: fpoc0001@glo.regionh.dk)

Abstract

Objective: A large, population-based case–control cohort was used to test the hypothesis that glutamic acid decarboxylase-65 (GAD65) and islet antigen-2 autoantibodies (IA-2A) at birth predict type 1 diabetes.

Design and methods: The design was an individually matched case–control study of all Danish type 1 diabetes patients born between 1981 and 2002 and diagnosed before May 1 2004 (median age at diagnosis was 8.8 years). Dried blood spot samples collected 5 days after birth in the 1981–2002 birth cohorts and stored at -25°C were identified from 2023 patients and from two matched controls ($n=4042$). Birth data and information on parental age and diabetes were obtained from Danish registers. GAD65A and IA-2A were determined in a radiobinding assay. *HLA-DQB1* alleles were analyzed by PCR using time-resolved fluorescence.

Results: GAD65A and IA-2A were found in 70/2023 (3.5%) patients compared to 21/4042 (0.5%) controls resulting in a hazard ratio (HR) of 7.49 ($P<0.0001$). The HR decreased to 4.55 but remained significant ($P<0.0003$) after controlling for parental diabetes and *HLA-DQB1* alleles. Conditional logistic regression analysis showed a HR of 2.55 ($P<0.0001$) for every tenfold increase in the levels of GAD65A and IA-2A. This HR decreased to 1.93 but remained significant ($P<0.001$) after controlling for parental diabetes and *HLA-DQB1* alleles.

Conclusion: These data suggest that GAD65A and IA-2A positivity at birth are associated with an increased risk of developing type 1 diabetes in Danish children diagnosed between 1981 and 2004.

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Introduction

Type 1 diabetes becomes clinically apparent after a preclinical period of varying length, during which immune-mediated destruction reduces the β -cell mass. This preclinical period varies but appears to be more rapid in younger individuals (1). Immune-mediated type 1 diabetes is thought to be determined by the actions, and possible interactions, of multiple genetic and environmental factors. At least half of the genetic risk is determined by alleles of the human leukocyte antigen (*HLA*) genes: *DQA1*, *DQB1*, and *DRB1* (2, 3). The rest is determined by non-*HLA* loci (2, 3). It is still unknown, if, when and what kind of environmental factors initiate autoimmune β -cell destruction. Viral infections, nutritional, or other factors might initiate the type 1 diabetes pathogenetic process already *in utero* (4, 5) or postnatally (reviewed in (6)). Reflecting β -cell autoimmunity and possibly destruction, autoantibodies

are often detected against glutamic acid decarboxylase-65 (GAD65 or GAD2), islet antigen-2 (IA-2), Zn transporter 8 (ZnT8 or SLC30A10), or insulin, alone or in combination (7). The risk for type 1 diabetes increases with an increasing number of autoantibodies, and one or more autoantibodies are detected in about 90% of newly diagnosed type 1 diabetes patients (4, 8).

Because the autoimmune process contributing to the development of type 1 diabetes may be initiated long before the appearance of clinical symptoms (9), ideally effective prediction and intervention strategies should be applied as early as possible. It is still not established whether the presence of islet autoantibodies at the time of birth impacts the development of type 1 diabetes. In recent reports, islet autoantibodies were found to be either protective (10), predictive (4), or without (11) impact on the development of type 1 diabetes.

Current prospective studies of birth cohorts have ascertained only a limited number of new patients per

year and are expected to take several years to provide sufficient statistical power. Furthermore, in the BABY DIAB (12) and the TRIGR (13) studies, only children with first degree relatives with type 1 diabetes are included but <15% of new onset patients belong to this category.

Epidemiological studies indicate that perinatal factors such as gestational infections, pre-eclampsia, birth weight (BW), and maternal age affect the risk for type 1 diabetes (6, 14, 15). However, in a recent Danish study, no significant correlation between BW, maternal age, and type 1 diabetes risk was detected (16). Owing to the complex nature of type 1 diabetes pathogenesis, combination of immunological and demographical parameters in a large population-based case-control study may improve the identification of factors that predict type 1 diabetes.

The aim of the present study was to estimate the effect of GAD65A and IA-2A at the time of birth on type 1 diabetes risk up to 23 years of age. Interaction between islet autoantibody status and risk alleles (*HLA-DQB1*), BW, birth length (BL), gestational age (GA), parental age and diabetes status, and age at diabetes onset is tested.

Materials and methods

Samples

The study was an individually matched population-based case-control study (17). All type 1 diabetes cases born in Denmark between January 1 1981 and December 31 2002, and diagnosed at 23 years or younger, were identified from the National Patient Register. These data were validated against an independent registry (DSBD, www.DSBD.dk) (17). A total of 2023 cases with available dried blood spots (DBSs) samples were identified. The median age at diagnosis was 8.8 years (range 1–22 years). Two control individuals matched by date and place of birth were selected using the databank of DBSs (see below). Control individuals were required to be alive and free of diabetes at the date of diagnosis of the matching cases. Information on parental diabetes status (at the time of delivery), including type of diabetes, was obtained from the National Patient Registry, and information on birth data (BW, BL, and GA) was obtained from the Danish Birth Register.

Surplus DBS samples collected for the Danish neonatal screening program have since 1981 been stored at -25°C in a central repository (18). Samples in the repository were organized by date of birth and place of birth. The two samples placed closest to a case sample were selected as control samples matched by date- and place of birth. For the present study, two 3.2 mm DBS punches per case and control were available for analysis of islet autoantibodies.

The protocol was approved by the Danish ethical committee and the Danish Data Protection Agency. The study was conducted according to the principles expressed in the Declaration of Helsinki.

Autoantibody assays

GAD65A and IA-2A were determined in a previously described radiobinding assay (17). The DBSs were limited in availability, which made it necessary to combine ^{35}S -methionine-labeled GAD65 and IA-2 in one assay similar to previous reports (19). Islet autoantibody levels were measured in counts per minute. Relative units (RU) were calculated as described in detail elsewhere (20). Samples from matched cases and controls were always measured in the same assay run. In the present and in previous (19, 21) investigations of islet autoantibodies eluted from DBSs, the sensitivity for GAD65Ab and IA-2Ab assays was assessed in 1950 consecutively diagnosed (May 2005–September 2008) type 1 diabetes patients from all over Sweden. Using a cut-off value at 31 RU/ml (99.5 percentile for control population of Swedish children), we found that 1317/1950 patients had GADA, i.e. representing 68% diagnostic sensitivity. In the IA-2A assay, we used a cut-off value of 5 RU/ml (99.5 percentile for control population of Swedish children) and found that 1465/1950 patients had IA-2A representing 75% sensitivity. Our laboratory participates in the Diabetes Autoantibody Standardization Program (DASP) (22), and in the 2007 serum exchange, the workshop sensitivity and specificity for GAD65Ab were 82 and 96% and the workshop sensitivity and specificity for IA-2Ab were 65 and 99% respectively.

Genotyping

Time-resolved fluorometry was used for identification of *HLA-DQB1* alleles (02, 0301, 0302, 0304, 0602, 0603, and 0604) as described in details (23).

Statistical analysis

We used conditional logistic regression for matched sets to analyze the data (SAS proc phreg). Because controls were sampled as being alive at the date of diagnosis of the case, the odds ratios from the analysis are estimates of hazard ratios (HRs) for type 1 diabetes.

Values of antibody measurements were $\log_{10}$ transformed in order to provide estimates of the effect of a tenfold increase of the antibody level.

Since case and control samples were matched by date of birth and hence age, date of sampling, and storage time, the effect of these variables cannot be assessed in this study. Interaction between autoantibody levels and these variables could in principle be assessed, but we have no reason to suspect that calendar time or storage

time affects autoantibody levels differentially between cases and controls. Additional perinatal and demographic factors (BW, BL, GA, parental age, parental diabetes, and gender), and genotypes, were included into multiple regression models.

Confounders

Perinatal factors (BW, BL, and GA), demographic factors (parental age and gender), parental type 1 diabetes, and *HLA-DQB1* genotype were considered possible confounders for the effect of islet autoantibodies on type 1 diabetes risk. *Interaction models*: the fact that the origin of islet autoantibodies detected in newborns most probably is of maternal origin was the rationale for testing for interaction between the effect of maternal type 1 diabetes and the presence of islet autoantibodies on type 1 diabetes risk. Separate effects for mothers with and without type 1 diabetes were considered.

Results

A total of 70/2023 (3.5%) of the cases were positive in the GAD65A and IA-2A assays (HR = 7.49, $P < 0.0001$). No significant change in frequency of antibody positivity and/or titers (measured in RU) by storage time could be detected (data not shown). The frequency of mothers and fathers with type 1 diabetes in the positive compared to negative islet autoantibody cases and controls is shown in Table 1. A total of 27 cases had mothers and 2 cases had fathers with type 1 diabetes compared to 8 and 1 respectively among the islet autoantibodies positive controls (n.s.). The HR 7.49 for positive islet autoantibodies status at birth decreased to HR 5.75 when controlling for parental diabetes. More islet autoantibody-positive cases, 27/70 (39%) than 27/1957 (1.4%) islet autoantibody-negative cases ($P < 0.0001$), had a mother with type 1 diabetes.

Gender had no significant effect on the risk of developing type 1 diabetes. We also tested whether high-risk *HLA-DQB1* alleles were confounders. The HR decreased to 4.55 but remained significant ($P < 0.0003$) after controlling for parental diabetes and *HLA-DQB1* alleles.

The conditional logistic regression analysis showed a HR of 2.55 for every tenfold increase of RU in the GAD65A and IA-2A assays ($P < 0.0001$). We next analyzed whether perinatal and demographic factors modified the latter HR (Table 2). Not only maternal (HR = 4.2; $P < 0.0001$) but also paternal (HR = 7.3; $P < 0.001$) diabetes patients were significant predictors, and also maternal age showed a significant effect (HR = 1.2/10 years; $P < 0.03$). Including parental type 1 diabetes and maternal age into the model reduced the type 1 diabetes risk from HR 2.55 to HR 2.23; however, the risk remained significant ($P < 0.0001$). Including both *DQB1* alleles as well as parental diabetes and maternal age into the model reduced the increased type 1 diabetes risk to HR 1.93, still indicating that islet autoantibody positivity had an independent risk ($P < 0.001$). There was no difference in autoantibody (aab) positivity in relation to age of type 1 diabetes diagnosis. In total, 57% of aab-positive cases were younger than 10 years of age (20% < 5 years, 37% 5–9 years), compared to all 1957 aab-negative cases, where the age distribution was similar: 58% of cases were diagnosed below 10 years of age (24% < 5 years, 34% 5–9 years).

Discussion

The present study shows that GAD65A and IA-2A positivity at birth were associated with a 7.5-fold increased risk for developing type 1 diabetes in this cohort of Danish children. Every tenfold increase in GAD65A and IA-2A levels at birth increased the risk for development of type 1 diabetes by a factor 2.55. Thus, both presence and titers of GAD65A and IA-2A had a strong risk-increasing effect. Maternal type 1 diabetes

Table 1 Distribution of parental diabetes status per autoantibody status (in combined assay) and per case/control status. Diabetes status is subdivided into numbers of mothers and fathers with type 1 diabetes, with other types of diabetes (type 2 diabetes, latent autoimmune diabetes in adults (LADA), gestational diabetes), unspecified diabetes (uncertain of diabetes type), or without diabetes (no).

Diabetes status	Autoantibody status (combined)							
	Cases				Controls			
	Positive		Negative		Positive		Negative	
	Mothers	Fathers	Mothers	Fathers	Mothers	Fathers	Mothers	Fathers
Type 1	27	2	27	120	8	1	14	37
Other	3	1	21	25	1	0	36	44
No	40	67	1904	1804	12	20	3969	3936
Unspecified	0	0	2	5	0	0	2	4
Unknown	0	0	3	3	0	0	0	0
Total (n)	70	70	1957	1957	21	21	4021	4021

Altogether (cases + controls) there were 6069 mothers and 6069 fathers.

Table 2 Inclusion of perinatal data and parental diabetes. The variables in the table may be responsible for some of the effects on type 1 diabetes risk falsely ascribed to the presence of autoantibodies, i.e. may be confounders.

Variables	Hazard ratio (HR)	95% CI	P<
Tenfold increase in RU autoantibodies	2.23	1.66–3.01	0.0001
Birth length – per cm	0.91	0.97–1.04	0.91
Birth weight – per kg	1.03	0.87–1.23	0.71
Gestational age – per week	1.00	0.95–1.04	0.84
Maternal age – per 10 years	1.22	1.02–1.489	0.03
Paternal age – per 10 years	0.93	0.80–1.09	0.37
Sex, male versus female	1.05	0.93–1.20	0.42
Maternal type 1 diabetes	4.23	2.29–7.81	0.0001
Paternal type 1 diabetes	7.31	4.71–11.35	0.0001

By inclusion into the statistical model, the individual increases/decreases in type 1 diabetes risk belonging to each variable are listed including the level of significance (*P* value) and confidence interval (CI).

was a confounder, but it was only responsible for approximately one-fifth of the risk increase associated with GAD65A and IA-2A presence. That the presence of islet autoantibodies at the time of birth may be predictive for later type 1 diabetes development was suggested by Lindberg *et al.* (4), who studied cord blood samples from 85 children who later developed type 1 diabetes and 321 control subjects. Our approach to test the validity of the previous observation (4) was to utilize the unique repository of DBS samples collected for the Danish neonatal screening program since 1981 (17, 18). This repository, unique in that the DBSs have been stored at -25°C , made it possible to study samples from 2023 type 1 diabetes cases and 4046 matched controls. The present study is to our knowledge the first to show in a large, validated, and population-based case–control investigation that islet autoantibodies at birth were associated with increased type 1 diabetes risk later in life.

Do the islet autoantibodies identified in the 5-day-old newborns have maternal origin or are they produced by the newborn themselves? It is well known that during second trimester of pregnancy, maternal immunoglobulin is actively transported across the placenta to the fetus (24). Antibody transport accelerates during the last trimester until birth, and IgG concentrations in the fetus exceed those of the mother (25). Neither maternal IgA nor IgM are transferred to the fetus. In the present study, detected GAD65A and IA-2A were mostly of the IgG subclass as Sepharose Protein A was used to separate free from antibody-bound labeled autoantigen. From vaccination studies (26), it is known that the ability of newborns to generate IgG responses increases over the first year, but adult responses to protein antigens are not achieved until 12–24 months of age. We assume that the GAD65A and IA-2A we measured were transferred from the mother to the child. It is therefore possible that subclinical islet autoimmunity in the pregnant mother may explain the increased risk for type 1 diabetes in her offspring.

Islet autoantibodies are strongly associated with HLA-conferred diabetes susceptibility both in families and in the general population (27). We asked whether autoantibody positivity is a surrogate marker of HLA-defined diabetes predisposition in the current study population. It is likely that the autoantibody-positive non-diabetic mothers carry HLA risk genotypes for type 1 diabetes at a higher frequency than the autoantibody-negative mothers. Accordingly, the autoantibody-positive mothers may also transfer such genotypes to their child resulting in an increased diabetes risk in the offspring. The type 1 diabetes risk ascribed to the presence of GAD65A and IA-2A was reduced but still remaining significant when *HLA-DQB1* genotypes were included into the model. It cannot be excluded that other genetic factors, with lower risk, may contribute to the risk of type 1 diabetes in the offspring to islet autoantibody-positive mothers. We conclude that the presence of islet autoantibodies at the time of birth predicts type 1 diabetes risk regardless of HLA risk genotypes and maternal diabetes.

In the present study, only one-fifth of the increase in disease risk related to the presence of GAD65A and IA-2A was actually related to maternal diabetes. However, GAD65A- and IA-2A-positive cases without diabetic mothers may be the most intriguing observation. Follow-up studies of these mothers will be of interest as gestational islet autoantibodies may increase the risk for *post partum* type 1 diabetes (28, 29). In these mothers, *in utero* infections (30) or other stressors (14) could either have had direct impact on β cells or may have caused β -cell damage by initiating a strong proinflammatory immune response. Furthermore, about 50% of those children whose mothers have type 1 diabetes were still GAD65A and IA-2A negative at the time of birth. Mothers diagnosed with type 1 diabetes have variable duration of their diabetes, and may become negative for autoantibodies prior to or during their pregnancy (31).

A possible weakness to our study is that we may in particular detect cases that had an early age at diagnosis. However, the GAD65A- and IA-2A-related effect on type 1 diabetes risk was found to be similar in children below and above 10 years of age at type 1 diabetes onset. Proportions of GAD65A- and IA-2A-positive cases with young diagnosis out of all GAD65A- and IA-2A-positive cases, and of young GAD65A- and IA-2A-positive cases out of all cases, were similar. In the DIPPP study (27), the majority of children who developed first multiple islet autoantibodies and then type 1 diabetes had an appearance of one or more islet autoantibody below the age of 5 years. In the DAISY study (11), no association could be shown between the presence of islet autoantibodies at birth and type 1 diabetes risk. In the BABY DIAB study (10), in contrast, the presence of maternal islet autoantibodies was suggested as protective against type 1 diabetes before the age of 8 years. Insulin autoantibodies (IAA)

persisting after the age of 8 months of age (12), on the other hand, were associated with an increased type 1 diabetes risk. Owing to limited DBS material, we were also not able to analyze IAA in the present study. Much larger cohort studies will be needed to further analyze the risk of islet autoantibodies at the time when DBSs are obtained for perinatal screening of monogenic diseases. In such studies, it will also be important to obtain data on maternal islet autoantibodies as well as *HLA* alleles and non-*HLA* genetic risk factors (2, 32). The individually matched case–control design implies that control subjects were selected as not having diabetes at the date of diagnosis of the corresponding case. None of the control subjects developed type 1 diabetes in the follow-up period; nevertheless, we cannot exclude some subjects of the control group, which may develop type 1 diabetes later in life. However, this is unlikely to affect the overall conclusion of the study.

In the present case cohort, specificity was very high, i.e. seven times as many future type 1 diabetes patients were GAD65A and/or IA-2A positive at birth compared to the control group. Because the number of cases and controls in our study population does not correspond to the real distribution in a background population (i.e. the ratio of cases to controls in the study is much higher), we are not able to draw conclusions for how specific and sensitive the presence of GAD65A and IA-2A at birth were for prediction of type 1 diabetes in the general population.

In conclusion, the present study represents a large population-based case–control study demonstrating that GAD65A and IA-2A positivity at birth suggest an increased risk of developing type 1 diabetes at any age before 23 years.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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