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**Relationship between ZnT8Ab, the *SLC30A8* gene and disease progression in children with newly diagnosed type 1 diabetes**

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**Abstract**

Autoantibodies against the newly established autoantigen in type 1 diabetes, zinc transporter 8, ZnT8, are presented as two types, ZnT8RAb and ZnT8WAb. The rs13266634 variant of the *SLC30A8* gene was recently found to determine the type of ZnT8Ab. The aim of this study was to explore the impact of this genetic variant and the ZnT8Ab on the residual beta-cell function during disease progression the first year after disease diagnosis in children with newly diagnosed type 1 diabetes. This cohort consists of 257 children aged <16 years, all patients were newly diagnosed with type 1 diabetes. A Boost-test was carried out at 1, 6, 12 months to characterize the residual beta-cell function. Carriers of the CC and CT genotypes of the rs13266634 SNP of the *SLC30A8* gene had higher stimulated C-peptide levels the first year after onset compared to the TT genotype group (29%,  $p=0.034$ ). CC genotype carriers were highly associated with the presence of ZnT8RAb subtype during disease progression (compared with TT,  $p<0.0001$ ). On the other hand the TT genotype was associated with the presence of ZnT8WAb subtype during disease progression (compared with CC,  $p<0.0001$ ). The C allele of the *SLC30A8* gene is associated with preserved beta-cell function in type 1 diabetes patients. The genetic determination of the rs13266634 variant on the ZnT8Ab specificity is sustained the first 12 month after diagnosis of type 1 diabetes in a pediatric cohort.

## Introduction

The T-cell mediated beta-cell destruction in type 1 diabetes is directed against beta-cell antigens with insulin, GAD-65, and islet cell antigen 512 being the classical and well-known ones. These antigens together identify 80% of the patients at clinical diagnosis or at risk of developing type 1 diabetes (1). Recently the zinc transporter 8, ZnT8, was established as a new major autoantigen in type 1 diabetes (1). Several studies (2, 3) report a strong genetic association between a non-synonymous single nucleotide polymorphism, rs13266634, of the ZnT8 encoding gene, *SLC30A8*, and the type of ZnT8Ab (either arginine (Arg/R) or tryptophan (Trp/W) recognising antibodies). This genetic determination of an autoimmune reaction has not been described for any of the other diabetes-related autoantibodies, and it is not known if it is associated with differences in disease progression/severity or whether this specificity is stable over time.

Variants of different non HLA-diabetic risk genes have been shown to influence beta-cell function up to 12 months after diagnosis. Previously, we reported the effect of the insulin VNTR region on the residual beta-cell function and the level of insulin autoantibodies during disease progression in type 1 diabetes children (4). Furthermore, two classical type 2 diabetes genes, the Kir6.2 and the PPARgamma were found to influence glycemic control during disease progression in type 1 diabetes patients (5, 6). No association of the ZnT8Ab's or the rs13266634 variant with the reduced beta-cell function in type 1 diabetes has been reported. The rs13266634 variant has, however, been reported to be associated with the development of T2D (7), and has been associated with reduced first phase insulin release following an intravenous glucose load in a glucose-tolerant population (8).

The objective of this study was to investigate 1) the association of the rs13266634 variant of the *SLC30A8* gene and the ZnT8Ab's with the residual beta-cell function, 2) the stability of the epitope specific ZnT8Ab's during disease progression, and 3) whether HLA risk groups were significantly associated with the ZnT8Ab titer levels in a cohort of children with new onset type 1 diabetes.

## **Subjects and Methods**

### ***Study populations from The Hvidoere Study Group on Childhood***

The study population was collected through The Hvidoere Study Group on Childhood Diabetes and is described in Mortensen et al 2009 (9). The cohort included 126 girls and 131 boys, 84% of the patients were white Caucasian, and age at clinical diagnosis was  $9.1 \pm 3.7$  years (mean  $\pm$  SEM), BMI  $16.5 \pm 3.2$  kg /m<sup>2</sup>, and HbA<sub>1c</sub>  $11.2 \pm 2.1\%$  at the time of diagnosis. DKA ( $\text{HCO}_3 \leq 15$  mmol/l and/or pH  $\leq 7.30$ ) was present in 20.7% of the cases at the time of diagnosis.

Exclusion criteria were: suspected non-type 1 diabetes (type 2 diabetes, maturity-onset diabetes of the young (MODY) or secondary diabetes), decline of enrolment into the study by patients or parents, and patients initially treated outside of the centres for more than 5 days. There were no significant differences with respect to gender distribution, age, anthropometric data, HbA<sub>1c</sub> at diagnosis, ethnicity or family history of diabetes between patients included and patients not included into the study (data not shown). The diagnosis of type 1 diabetes was according to the World Health Organization criteria. The study was performed according to the criteria of the Helsinki II Declaration and was approved by the local ethic committee in each centre. All patients, their parents or guardians gave informed consent.

**Stimulated C-peptide test**

Residual beta-cell function (C-peptide) in response to a Boost-test (6 ml/kg (max: 360 ml) of Boost/Sustacal (Mead Johnson, Evansville, IN, USA; 237 ml = 8 FL OZ contains 33 g carbohydrate, 15 g protein and 6 g fat, a total of 240 kcal)) was followed 1, 6 and 12 months ((± 1 week) after diagnosis in all 257 children with newly diagnosed type 1 diabetes. Blood was drawn 90 minutes after ingestion of the Boost™ drink. Serum samples were labelled and frozen at – 20 °C until shipment on dry ice. C-peptide was analysed centrally. Samples were thawed only once for RIA determination.

Plasma C-peptide was analysed by a fluoroimmunoassay (AutoDELFIA™ C-peptide. Analytical sensitivity: better than 4.97 pmol/l CV 5%).

**Typing of the *SLC30A8* gene**

Genotyping of the rs13266634 variant of the ZnT8 gene, *SLC30A8*, was done both at KBioscience using an in-house KASPar assay system and at Steno Diabetes Center, Denmark, using a predesigned TaqMan assay (Applied Biosystems, Foster City, CA, USA) on a TaqMan 7900HT (Applied Biosystems, Foster City, CA, USA). The concordance rate between the two assays was 97%.

**HLA**

Typing of the HLA-class II DRB1 locus was performed by direct sequencing of exon 2 of DRB1 according to Immuno Histocompatibility Working Group. The HLA risk groups were defined as follows: high risk (*DRB1* 03/04, 04/04), moderate risk (*DRB1* 03/03, 04/08), and low risk (all other *DRB1* genotype combinations).

**Diabetes related autoantibodies (IAA, ICA, GADA, IA-2A and ZnT8Ab)**

**IAA.** Insulin antibodies were measured by a modification of the method described by *Williams et al.* [10]. The cut-off limit for positivity is 1.56 RU, representing the 99<sup>th</sup> percentile in a group of 371 non-diabetic subjects.

**ICA.** Islet cell antibodies (ICA) of the IgG class were detected by indirect immunofluorescence using commercial Primate Pancreas slides from INOVA. The sera were screened at a dilution of 1:2 and FITC-labelled anti-human IgG (Dako, Copenhagen, Denmark) was used as conjugate.

**GADA.** Antibodies to the 65kD isoform of glutamic acid decarboxylase (GADA) were quantified by a direct radioimmunoassay (Diamyd Anti-GAD-65 RIA; Diamyd Diagnostics, Stockholm, Sweden) according to the protocol provided by the manufacturer. Sera were run in duplicate, and the results were read on a gamma counter (Wizard 1470; Wallac/PerkinElmer, Turku, Finland) and calculated from a standard curve. The cut-off limit was 9.5 units/ml and the intra- and interassay coefficients of variation were 2.4% and 3.6%, respectively.

**IA-2A.** Antibodies to the protein tyrosine phosphatase related IA-2 molecule (IA-2A) were analysed with a radiobinding assay as previously described [11]. The results were expressed as relative units (RU) based on a standard curve run on each plate using an automated calculation program (MultiCalc; Wallac). The limit for IA-2A positivity (0.77 RU) was set at the 99<sup>th</sup> percentile in 374 non-diabetic children and adolescents. The interassay coefficient of variation was < 12%. This assay had a disease sensitivity of 72% and a specificity of 100% based on the 2005 Diabetes Autoantibody Standardisation Programme (DASP) workshop.

**ZnT8Ab**

**Subcloning of the C-Terminal construct (Arg 325) ZnT8R from pCDNA3.1 to generate pThZnT8R**

The original C-terminal cDNA construct coding for Arg at position 325 [1] was a kind gift from John C Hutton (Barbara Davis Center for Childhood Diabetes, University of Colorado at Denver and Health Sciences Center, Aurora, CO, USA). The insert was subcloned into the pTnT<sup>TM</sup> vector (Promega, Madison, USA) as described [12, 13]. The ZnT8W variant was generated by site-directed mutagenesis and confirmed by DNA sequencing [12,13].

**Coupled *in vitro* transcription/translation of cDNA for ZnT8R and ZnT8W.**

The coupled *in vitro* transcription/translation of the ZnT8R in the pCDNA3.1 vector and the two new plasmids, pThZnT8R and pThZnT8W were performed as follows. The reaction mixture of 2 µg ZnT8R in the pCDNA3.1 vector, pThZnT8R or pThZnT8W, 50µL TNT® rabbit reticulocyte lysate, 4µL TNT® reaction buffer, 2µL amino acid mixture minus Methionine, 2 µL RNasin® Ribonuclease inhibitor (Promega), 2µL SP6 RNA Polymerase, 4µL <sup>35</sup>S-methionine (Amersham Int., Amersham, Bucks., UK, >1,000Ci/mmol), and nuclease-free water to a final volume of 100µL was incubated for 90 min at 30°C with shaking (300rpm in Eppendorf Thermomixer comfort, Eppendorf, www.eppendorf.com). The translation product was immediately subjected to gel filtration on Illustra<sup>TM</sup> NAP-5 Columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and percent radioactivity incorporated into protein was counted (1450 MicroBeta TriLux Microplate Scintillation-Luminescence Counter, PerkinElmer Life and Analytical Sciences, Shelton, CT, USA, www.perkinelmer.com). The percent radioactivity for ZnT8R in the pCDNA3.1 vector was 10 ±3% (mean±SD) compared to >40% for each new plasmids including pThZnT8R and pThZnT8W. The translation



product corresponding to the most C-terminal part of ZnT8 protein (aa268-aa369) for ZnT8R and ZnT8W was demonstrated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

#### **Autoantibody Radio-Binding Assay for ZnT8R and ZnT8W**

The RBA for the individual variants were performed separately with 5 $\mu$ L of sera essentially as described [12]. Duplicate serum samples were incubated over night at 4°C with 60 $\mu$ L labeled antigen diluted in antigen buffer (150 mM NaCl, 20 mM Tris, pH 7.4, 0.15% Tween20, 0.1% BSA) at a final concentration 425 $\pm$ 25 cpm/ $\mu$ L using MicroWell™ plates (Nunc, www.nalgenunc.com). Antibody bound was separated from free labeled antigen with Protein A Sepharose (20%) and the radioactivity determined in a beta-counter (a 1450 MicroBeta TriLux Microplate Scintillation-Luminescence Counter (PerkinElmer Life and Analytical Sciences, Shelton, CT, USA, www.perkinelmer.com). The results were expressed in in-house units using two different standard curves of type 1 diabetes-sera positive for each of ZnT8R and ZnT8W respectively. Positivity for ZnT8RAb was defined as titer values  $\geq$  60 U/ml and for ZnT8WAb  $\geq$  58 U/ml. Out of 257 patients 251 completed ZnT8Ab measurements.

#### **Statistics**

The association between the rs13266634 variant and the ZnT8Ab level was determined by logistic regression with age, sex, HLA risk groups and DKA at onset as confounding factors at all three visits (1, 6 and 12 months after onset). Stimulated C-peptide (logarithmic) and insulin dose-adjusted HbA<sub>1c</sub> (IDAA1c) [ 12] were analyzed as dependent variables in two separate multiple regression models by a compound symmetrical repeated measurement model of all time points with rs13266634 genotype, ZnT8W/RAb levels, sex, age, positivity for other diabetes-related autoantibodies

(insulin autoantibodies (IAA), glutamic acid decarboxylase (GADA), islet cell autoantibodies (ICA), insulinoma-associated antigen-2 (IA-2A)) and HLA risk groups (high (DRB1 03/04, 04/04), moderate (DRB1 03/03, 04/08), low (all other genotype combinations)) as co-variables. P values below 0.05 were considered statistical significant.

## **Results**

**Frequency of ZnT8Ab.** 1 month after diabetes onset 68% of the patients tested positive for either one or both of the ZnT8Ab variants (ZnT8RAb and/or ZnT8WAb). At 6 and 12 months after onset the overall positivity was non-significantly decreased to 63% and 61%, respectively. The frequency of patients tested positive for ZnT8RAb was highest throughout the study period compared to frequency of patients positive for ZnT8WAb ( $p < 0.0001$ ) (Figure 1),

In the same Hvidoere cohort we previously diagnosed 24 out of 261 (9.2%) pancreatic antibody negative (ICA, GADA, IA-2A) children with new onset type diabetes. When we include the measurement of ZnT8Ab, 6 patients from this autoantibody negative subgroup were identified as ZnT8Ab positive (5 patients ZnT8RAb positive and 1 patient positive for both ZnT8Ab variants), reducing the frequency of Ab negative patients by 25% (Table 1) in the whole cohort.

### **ZnT8Ab titer level associates with age-at-onset**

The frequency of ZnT8RAb positive was significantly higher ( $p = 0.03$ ,  $0.03$  and  $0.02$  at 1, 6 and 12 months after onset, respectively) in patients above 5 years ( $n = 133$ ) at onset compared to the patients below 5 years ( $n = 20$ ) at onset (Table 2). By contrast the prevalence of the ZnT8WAb was independent of age-at-onset (data not shown).

***SLC30A8* rs13266634 genotype in relation to residual beta-cell function**

In a co-dominant model (comparing differences in stimulated C-peptide between all three genotype groups) carriers of the *SLC30A8* rs13266634 TT genotype had significantly lower stimulated C-peptide 1 month after onset compared to CC genotype carriers (est.: 0.644 pmol/l,  $p=0.049$ ). The CT and CC genotype carriers showed similar levels of stimulated C-peptide which allowed us to use a dominant model with respect to the C allele (CT+CC versus TT) in a mixed-model including all visits 1, 6 and 12 months after onset. The TT genotype carriers showed 29% lower stimulated C-peptide levels compared to the CT+CC genotype carriers during the study period (est.: 0.71 pmol/l,  $p=0.034$ ) (Figure 2). Adding a gene\*visit interaction term to model showed no differential gene effect over time ( $p=0.53$ ).

**IAA, GADA, ICA, IA-2A and ZnT8Ab in relation to glycemic control and residual beta-cell function**

The observed progressive decline in ZnT8Ab during disease progression was concurrent with the decline in stimulated C-peptide (Figure 3). There was, however, no association between positivity or titer levels of ZnT8Ab and the residual beta-cell function as estimated by stimulated C-peptide at any time points during disease progression ( $p=0.21$  (Trp) and  $p=0.95$  (Arg)). Neither can the ZnT8Ab measured 1 month after type 1 diabetes diagnosis predict the stimulated C-peptide level 12 months after disease onset ( $p=0.30$  (Trp) and  $p=0.19$  (Arg)). The same lack of predictive value was also evident for the ICA and IA-2A antibodies (data not shown). On the contrary the IAA and GADA antibodies at 1 month can predict stimulated C-peptide 12 months after clinical diagnosis (est.: 32% lower C-peptide,  $p=0.02$ , est.: 39% lower C-peptide,  $p=0.0004$ ).

GADA at 1 month also predicts Hba1c at 12 months (est.: 0.48%,  $p=0.012$ ), while none of the other diabetes-related autoantibodies predicts HbA1c at 12 months.

#### **ZnT8Ab titer levels according to HLA risk genes**

The ZnT8RAB levels were significantly higher in carriers of the low HLA risk groups compared to carriers of the moderate and high HLA risk groups 1 and 6 months after onset ( $p=0.04$ ) and borderline significant at 12 months after onset ( $p=0.06$ ). The levels of the ZnT8RAB were comparable in the moderate and high risk groups (Figure 4), justifying a model in which carriers of the low HLA risk group is compared to the moderate and high HLA risk groups. There were no significant differences in the distribution of ZnT8WAb according to HLA risk groups (data not shown).

#### **Association between the rs13266634 gene variant and ZnT8Ab specificity**

The genotype distribution in this cohort (CC 46.8%, CT 41.7%, TT 11.5%) was in Hardy-Weinberg equilibrium. We find the rs13266634 SNP is highly associated with the ZnT8Ab type 1 month after disease onset and this association is sustained during disease progression (6 and 12 month after disease onset). The CC genotype carriers have significantly higher ZnT8RAB compared to the TT genotype carriers (1 month  $p<0.0001$ , est.: 370U/ml) (Figure 5A). The heterozygous carriers have intermediate values for both ZnT8Ab variants. The opposite picture was evident for the ZnT8WAb, where the TT carriers have a significantly higher ZnT8WAb compared to the CC genotype carriers (1 month  $p<0.0001$ , est.: 374U/ml) (Figure 5B). In patients only responding to either ZnT8RAB or ZnT8WAb the relationship with the rs13266634 variant was indeed stronger. A 4.6 fold higher ZnT8RAB frequency was seen among CC carriers compared to the CT carriers, while the frequency of ZnT8WAb was 15.5 fold higher among the TT carriers compared to CT carriers (Table 3).

## Discussion

This study suggests an association between the rs1326634 variant of the *SLC30A8* gene and the residual beta-cell function in children with new onset type 1 diabetes and a relation between the HLA risk groups and the titer of ZnT8RAb. Furthermore, a strong relation between the rs13266634 variant of the *SLC30A8* gene and the specificity of the ZnT8Ab was found. This is a newly described phenomenon involved for type 1 diabetes associated autoantibodies [2]. Our study supports this association and furthermore extends these observations into a pediatric population from time of disease onset and the following year.

In the present pediatric cohort, there is a non-significant decreasing trend of positivity for ZnT8Ab from onset to 12 months after diagnosis for both Arg and Trp recognizing autoantibodies (Figure 1). This is similar to the observed pattern of GADA, IA-2A and ICA in the same cohort [4], whereas IAA tends to increase from onset due to insulin treatment. A relation between age-at-onset and positivity for ZnT8RAb was previously indicated in a mixed cohort of children and adults, where the prevalence of ZnT8Ab was low in the very young children but increased dramatically from 3 years onward, peaked at 80% in late adolescence and tended to decline thereafter [2]. Our study supports this observation because the youngest patients (0-5 years) presented with and had the lowest ZnT8RAb levels during disease progression (Table 2). This difference suggests the peak of autoimmune response occurred before time of onset for this age group. Interestingly, this effect of age was not seen for ZnT8WAb levels.

When we analyzed ZnT8Ab in this cohort an additional 2.3% of the GADA, ICA and IA-2A negative patients were ZnT8Ab positive (Table 1). Lampasona and co-workers have previously identified an additional 1.4% of autoantibody negative subjects as

autoantibody positive when testing for ZnT8Ab in an adult-onset type 1 diabetes cohort [13]. In agreement with the previously reported 26% ZnT8Ab-positivity-rate among patients classified as autoantibody-negative [1] we find that 25% of the children classified as autoantibody negative in our study were ZnT8Ab positive, either for the Arg or Trp variant. Therefore in clinical practice the inclusion of ZnT8Ab may help to differentiate clinical phenotypes.

Our study suggests a functional impact of the rs13266634 variant of the *SLC30A8* gene on progression of the disease. Analyzing the gene effect in a multiple regression analysis including all three visits (1, 6 and 12 months) in the same model we found, that carriers of the TT genotype had significantly lower residual beta-cell function 12 months after disease diagnosis. This effect was, however, primarily due to the difference observed at 1 month (Figure 2). The rs13266634 has previously been shown to associate with T2D [7] and reduced first-phase insulin release in a glucose-tolerant population, although this effect appeared to be associated with the CC genotype [8]. At first this may seem contradictory, but assuming a reduced insulin secretory capacity in C-allele carriers, this may in type 1 diabetes turn out to be a beta-cell protective effect. Insulin is a major autoantigen in type 1 diabetes, so it seems plausible to hypothesize that reduced insulin secretory capacity associated with the C-allele and in the presence of insulin directed autoimmunity will reduce insulin antigen presentation, reduce beta-cell stress and slow down autoimmune beta-cell destruction. On the contrary, the T-allele associated with higher insulin secretory capacity should in the presence of insulin directed autoimmunity lead to higher insulin antigen presentation, beta-cell stress and autoimmune beta-cell destruction. In accordance with this

hypothesis, we find that TT-genotypes have lower residual beta-cell function compared to CC-genotypes.

A concurrent decline in stimulated C-peptide and ZnT8Ab titers (Figure 3) was also previously found in a smaller type 1 diabetes population (n=21), neither of the two studies found a correlation between ZnT8Ab and residual beta-cell function during the study period [17]. In our study, ZnT8Ab's at 1 month did not predict residual beta-cell function at 12 months, although we did find that the level of IAA and GADA at 1 month after diagnosis was associated with a more rapid loss of residual beta cell function. Together, these observations suggest that the ZnT8Ab's alone do not play a major role in the autoimmune destruction of the beta-cells, whereas IAA and GADA seem to do so.

The finding that carriers of the HLA low risk alleles have significantly higher ZnT8RAb 1 and 6 months after clinical onset (Figure 4), is most likely a spurious finding as similar was not observed for the other more pathogenically related autoantibodies, GADA and IAA.

The strong association between the rs13266634 variant and the two types of the ZnT8Ab throughout initial clinical disease progression suggests that epitope switching did not occur during the study period (Figure 5). That Trp recognizing ZnT8Ab was found in a few CC genotype carriers (encoding Arg) indicate, however, that the specificity is not 100% but the association between the genotype and ZnT8Ab subtype is complete when analyzing patients only presenting ZnT8RAb, or only presenting ZnT8WAb (Table 3).

Our study confirms the genotypic specificity of ZnT8Ab in a cohort of new onset type 1 diabetes children, and although we did not observe association or

predictive value of these autoantibodies on disease progression, the role of ZnT8 as an autoantigen in the pathogenesis of type 1 diabetes should be further explored, like insulin and GAD65 have been, in immune intervention trials with recombinant ZnT8 epitopes. The role of the rs13266634 variant on beta-cell function during disease progression in type 1 diabetes patients needs confirmation from studies of disease progression in other type 1 diabetes populations.

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**Captions**

Figure 1: Positivity of ZnT8Ab (either ZnT8RAb and/or ZnT8WAb) in children with type 1 diabetes 1, 6 and 12 months after disease onset. \* indicate significant difference in ZnT8Ab positivity between the two subtypes.

Figure 2: The stimulated C-peptide levels during disease progression according to a dominant gene model, the TT genotype carriers of the rs1326634 variant versus the CT+CC genotype carriers.

Figure 3: The concordant decline in stimulated C-peptide and the two ZnT8Ab subtypes, ZnT8RAb and ZnT8WAb, during disease progression.

Figure 4: The distribution of ZnT8RAb during disease progression according to *DRB1* HLA risk genes. The mean ZnT8RAb values $\pm$ sem are plotted against the three HLA risk genotype groups, low, moderate and high. \* indicate significance differences in ZnT8RAb level between genotype groups.

Figure 5: Association between the rs1326634 variant and the ZnT8Ab presentation during disease progression. A. The ZnT8RAb distribution according to CC, CT and TT genotype groups. B. The ZnT8WAb distribution according to these genotype groups.

**Appendix**

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