Original article

Title: Correlations between islet autoantibody specificity and the *SLC30A8* genotype with *HLA-DQB1* and metabolic control in new onset type 1 diabetes.

Running title: *SLC30A8* and ZnT8A in type 1 diabetes

Authors: Caroline Brorsson[1], Fariba Vaziri-Sani[2], Regine Bergholdt[1], Stefanie Eising[1], Anita Nilsson[2], Jannet Svensson[3], Åke Lernmark[2] and Flemming Pociot[1, 2, 4, *]

[1]Hagedorn Research Institute and Steno Diabetes Center, Gentofte, Denmark

[2]Department of Clinical Sciences, University Hospital Malmö, Lund University, Malmö, Sweden

[3]Glostrup University Hospital, Glostrup, Denmark

[4]Department of Biomedical Sciences, University of Copenhagen, Copenhagen, Denmark

[*]Corresponding author:

Flemming Pociot (fpoc@hagedorn.dk)

Hagedorn Research Institute

Niels Steensensvej 1

DK-2820 Gentofte

Denmark

Telephone: +45 44439371

Fax: +45 44437313

Word count abstract: 250

Word count main text: 2867

ABSTRACT

We hypothesised that the correlation between autoantibody specificity for the ZnT8 Arg325Trp isoforms and the type 2 diabetes associated rs13266634 may affect β-cell function at type 1 diabetes (T1D) onset. In order to study this, we tested 482 newly diagnosed diabetic probands and 478 healthy siblings from the Danish population-based T1D registry for autoantibodies to ZnT8 (ZnT8A) in addition to GAD65 and IA-2. The prevalence and titers of autoantibodies were correlated to genotypes for rs13266634 and *HLA-DQB1*, age at diagnosis (AAD) and insulin dose-adjusted HbA1c (IDAA1c), as a proxy for residual β-cell function. We replicated the correlation between rs13266634 genotypes and specificity for the ZnT8-Argenine (ZnT8R) and ZnT8-Tryptophan (ZnT8W) isoforms previously reported. ZnT8A overlapped substantially with GADA and IA-2A and correlated significantly with IA-2A prevalence ($p<2e-16$). No effect on AAD or IDAA1c was demonstrated for ZnT8A or rs13266634. We found a correlation between ZnT8R positivity and *HLA-DQB1*0302* genotypes ($p=0.016$), which has not been shown previously. Furthermore, significantly lower ZnT8R and GADA prevalence and titres was found among probands with AAD<5 years (prevalence: $p=0.004$ and $p=0.0001$; titers: $p=0.002$ and $p=0.001$, respectively). The same trend was observed for IA-2A and ZnT8W, however the difference was non-significant. Our study confirms ZnT8 as a major target for autoantibodies at disease onset in our Danish T1D cohort of children and adolescents and we have further characterised the relationship between autoantibody specificity for the ZnT8 Arg325Trp epitopes and rs13266634 in relation to established autoantibodies, age at diagnosis, measures of β-cell function and *HLA-DQB1* genotypes in T1D.

Keywords: ZnT8, *SLC30A8*, autoantibody specificity, *HLA-DQB1*, β-cell function

INTRODUCTION

Type 1 diabetes (T1D) is an immune-mediated disease of complex aetiology specifically targeted at the insulin producing pancreatic β-cells. Autoantigens directed against proteins such as insulin, glutamate decarboxylase 65 (GADA) and protein tyrosine phosphatase-like antigen IA-2 (IA-2A) have been demonstrated to be good markers for the immunological process that precedes clinical disease [1-3], however they are not specific enough to fully predict disease outcome. Recently a new autoantigen in T1D was identified as zinc transporter, member 8 (ZnT8) [4]. The protein is located in the membrane of insulin containing vesicles were it facilitates transport of zinc ions from the cytoplasm into the vesicles [5, 6], which is important for proper insulin storage and secretion. A single nucleotide polymorphism (SNP) in the gene *SLC30A8*, coding for ZnT8, was originally identified to confer risk of type 2 diabetes (T2D) in genome wide association scans [7-9]. The SNP rs13266634 causes an amino acid substitution on residue 325, from an arginine (Arg/R) encoded by the T2D associated C allele to a tryptophan (Trp/W) encoded by the minor T allele. Following the original report of ZnT8 autoantibodies (ZnT8A) in T1D, Wenzlau et al. identified autoantibodies against two epitopes in ZnT8 in diabetic individuals [10]. One epitope contained the arginine variant (ZnT8R) and the other the tryptophan variant (ZnT8W) encoded by rs13266634. Furthermore, it was demonstrated that the rs13266634 genotype determined the specificity for the individual antibody response, so that autoantibodies against the ZnT8R variant were more prevalent in CC homozygotes whereas autoantibodies against the ZnT8W variant were more prevalent in TT homozygotes. These findings have subsequently been replicated [11, 12]. The C allele of rs13266634 has been associated with functional effects in β-cells such as decreased insulin release [13, 14], proinsulin to insulin conversion [15], first phase insulin response

[16] and HbA1c levels [17]. We and others have been unable to demonstrate an association for rs13266634 with the risk of T1D [18-21]. Functional studies have demonstrated that INS-1E cells over-expressing ZnT8 had an increased insulin secretion after glucose stimulation [6]. Furthermore, rat islets and INS-1E cells stimulated with IL-1β displayed a down regulated expression of ZnT8 and cells over-expressing ZnT8 were more sensitive to cytokine induced apoptosis [22], suggesting a role for ZnT8 in the pathogenesis of T1D. In the present study we have investigated the prevalence of autoantibodies against the ZnT8 Arg325Trp isoforms and their correlations to rs13266634 in newly diagnosed Danish T1D probands and healthy siblings. The aim was also to test if ZnT8A or rs13266634 genotypes had an effect on age at diagnosis or insulin dose-adjusted HbA1c (IDAA1c) as a proxy for β-cell function at disease onset. Furthermore, we tested whether ZnT8R or ZnT8W could be correlated to specific *HLA-DQB1* genotypes.

METHODS

Samples

Type 1 diabetic probands and healthy siblings were collected by the Danish Study Group of Childhood Diabetes (DSBD). Probands with blood sampling within 3 months after onset were chosen. The date of onset was defined as the date of first insulin injection and diabetes duration thereafter was measured in months. A sample of siblings was chosen with similar age and sample year representing the entire study period. Blood samples from the same family were taken within 1 month in 90% of the families. There were 18 cases and 21 siblings excluded due to insufficient material for the study. Probands and siblings chosen were not necessarily from the same family, but all the siblings included have a sibling diagnosed with diabetes before the age of 19 with continued insulin treatment since

diagnosis. This resulted in 482 diabetic probands and 478 healthy siblings included in the study. The median age-at-onset was 10 years (range 0-18). The probands were divided into three age groups for further studies; age at diagnosis (AAD): below 5, 5 to 10 and above 10 years of age. The gender distribution was 53% boys among the probands and 56% boys among the siblings. The study was performed according to the Helsinki II Declaration and approved by the Danish Ethical Committee. Informed consent was given by all participants, their parents or legal guardians.

Antibody Assays

The ZnT8R pcDNA3.1 plasmid (kindly supplied by John Hutton, Barbara Davis Center for Childhood Diabetes, University of Colorado at Denver and Health Sciences Center, Aurora, CO) was used to excise the ZnT8R cDNA insert, which was ligated into the pTnT high efficiency vector (Promega, Madison, USA). Site directed mutagenesis was carried out with the Phusion TM site-directed mutagenesis kit (Finnzymes Oy, Espoo, Finland) to substitute the Arg for Trp at position 325. All cDNAs were sequenced. The pThZnT8R and pThZnT8W plasmids were subjected to coupled in vitro transcription translation to generate ^{35}S-methionine-labeled human recombinant ZnT8 proteins with the TNT® SP6 Coupled Reticulocyte Lysate System (Promega). Labelled ZnT8 was separated from free ^{35}S-methionine by gel filtration on Illustra™ NAP-5 Columns (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Incorporated radioactivity for ZnT8R in the pcDNA3.1 vector was 10±3% (mean+SD) compared to 26±3% for pThZnT8R and 27±2% for pThZnT8W, respectively. Autoantibodies were measured in standard radioligand binding assays. Antibodies against GAD65 and IA-2A were measured as previously described [23, 24]. Antibody positivity for ZnT8R, ZnT8W, GADA and IA-2A was defined as a value above 30

U/ml, 58 U/ml, 50 U/ml and 5 U/ml respectively, based on the 99th percentile in an independent control material consisting of blood donors from Malmö, Sweden. These antibody levels were used to divide individuals into antibody positive and antibody negative groups.

Genotyping

A total of 458 of the diabetic probands and 457 siblings had genomic DNA available and were genotyped for rs13266634 using TaqMan Allelic Discrimination assay (Applied Biosystems, Foster City, CA, USA) on the 7900HT sequence detection system. All genotypes were in Hardy-Weinberg equilibrium. 446 of the diabetic probands were genotyped for *HLA-DQB1* alleles by PCR using sequence specific primers, time-resolved-fluorometry [25] and lanthanide-labelled, short allele-specific oligonucleotide probes (detecting *0301, *0302, *0602, *0603, *0604, *02 and control) in a solution hybridization assay by DELFIA® (PerkinElmer, Turku, Finland). Probands were divided into three *HLA-DQB1* genotype groups; *DQB1*02*: representing 02_X/604; *DQB1*0302*: representing 302_2/604/X; Moderate and protective: representing 301_2/X; 304_2/X; 603_2/302/X; 604_301/X; X_X; 602_**.

Insulin Dose-Adjusted HbA1c

An insulin dose-adjusted HbA1c (IDAA1c) was calculated as actual HbA1c (%) + 4*insulin dose U/kg/24h [26] and was used to evaluate residual β-cell function. Only probands with values taken less than 1 year after diagnosis were considered for analysis (n=257). An IDAA1c below 9% has been demonstrated to correspond to an estimated maximal C-peptide level above 300 pmol/l [26, 27].

Statistical Analyses

Diabetic probands and siblings were classified as autoantibody positive or negative for each of the four autoantibodies tested according to the cut-off levels mentioned. All correlations between categorical variables (antibody prevalence, genotypes and AAD groups) were investigated using a χ-square test. A non-parametric analysis of variance test (Kruskal-Wallis rank sum test or Wilcoxon rank sum test) was used to compare autoantibody titers, IDAA1c levels, AAD as a continuous variable and diabetes duration between groups. All statistical analyses were performed in R (www.r-project.org).

RESULTS

We found that 61.2% (n=295) of the diabetic probands were positive for autoantibodies against either ZnT8R or ZnT8W compared to only 1.9% (n=9) of the healthy siblings (**Table 1**). These values corresponded to the data for IA-2A and GADA (**Table 1**). The proportion of autoantibody positive probands was raised from 85.3%, when only testing for IA-2A and GADA, to 89.6% when including ZnT8A. In the top part of **Table 2** positivity for the four autoantibodies was correlated to genotypes for rs13266634. We replicated the correlation between ZnT8R autoantibodies and carrying the C allele and likewise between ZnT8W autoantibodies and the T allele. Positivity for IA-2A and GADA was independent of rs13266634 genotypes. Genotypes for rs13266634 were obtained for 283 of the 295 probands with a positive ZnT8A response. Of these were 66 positive for ZnT8R only, 51 positive for ZnT8W only and 166 positive for both. When comparing the genotype distribution between the ZnT8R-only and the ZnT8W-only groups there was an even stronger correlation to the rs13266634 genotype (**Table 2,** middle part). Among the 59 that

carried the CC genotype 58 had a ZnT8R restricted response whereas all 25 carrying the TT genotype had a ZnT8W restricted response. In the group that carried the CT genotype 75.8% (25/33) were ZnT8W restricted. Among the 166 probands that reacted against both epitopes 44.0 % carried the CC genotype whereas 49.4% and 6.6% carried the CT and TT genotype, respectively (**Table 2,** bottom part).

Prevalence of ZnT8A Correlate to IA-2A but Not GADA

A substantial overlap was found between ZnT8A and the two other autoantibodies tested, GADA and IA-2A (**Figure 1**). There was a high correlation between IA-2A and both variants of ZnT8A, as 81% of the diabetic individuals that were positive for ZnT8A were also positive for IA-2A compared to 43.3% in the ZnT8A negative group ($p<2.2e-16$, **Table 3**). The same trend was found between GADA and ZnT8A, however not significant.

ZnT8R Prevalence and Titers are Higher in Children Diagnosed After 5 Years of Age.

The median age at diagnosis for the probands was 10 years (range 0-18). Probands were divided into three groups: age at diagnosis below 5 years (N=64), between 5 to 10 years (N=165) and over 10 years (N=252). We found that ZnT8A and GADA were more frequent among probands with an age at diagnosis above 5 years of age compared to those below 5 years of age ($p=0.017$ and $p=0.0001$ respectively, **Table 4**). However, when the two ZnT8A variants were analysed separately, the difference was only significant for the ZnT8R variant (**Figure 2A** and **2B**, ZnT8R: $p=0.004$ and ZnT8W: $p=0.12$). The same trend was found for IA-2A, however not significant (**Figure 2C**, **Table 4**). Furthermore, when analysing actual antibody titres between age groups we found generally higher titers of all four antibodies in children with an age-at-diagnosis above 5 years. This was again most

marked and reached statistical significance for ZnT8R and GADA (**Figure 3A-D**, p=0.002 and p=0.001 respectively). Different genotypes for rs13266634 did not affect age at diagnosis when analysed within the three age groups (p=0.40), or as a continuous variable (p=0.42), although there was a trend towards an allele-dosage effect for the C allele and a lower mean age at diagnosis.

Higher frequency of HLA-DQB1*0302 correlates to ZnT8R but not ZnT8W

The majority of the diabetic probands were genotyped for *HLA-DQB1* and were found to carry high risk genotypes, with the largest group being represented by *DQB1*0302* genotypes (65.6%). ZnT8R positivity correlated to a higher frequency of the *DQB1*0302* genotypes (p=0.016) whereas ZnT8W positivity was found to be independent of *HLA-DQB1* (**Table 5**, top part). ZnT8R-only responders also correlated to a higher frequency of *DQB1*0302* genotypes and lower frequency of the moderate and protective genotype group when compared to the ZnT8W-only responders (p=0.015 and p=0.020, respectively, **Table 5**, bottom part). As expected IA-2A positivity correlated strongly to a higher frequency of the *DQB1*0302* genotypes whereas GADA positivity correlated to a higher frequency of the *DQB1*02* genotypes, which has previously been reported [28] (**Table 5**, top part).

Insulin Dose-Adjusted HbA1c

We detected a trend towards a higher mean IDAA1c in the CC homozygote carriers of rs13266634 compared to the CT and TT genotype carriers, however this did not reach statistical significance (p=0.48, **Table 6**). No difference in diabetes duration was detected between the groups but age-at-diagnosis increased with the number of T alleles (p=0.049).

The same trend was observed in the full data set, however not significant (p=0.42). The presence of ZnT8A did not have an effect on mean IDAA1c (ZnT8R p=0.17 and ZnT8W p=0.32).

DISCUSSION

In this study we have shown that the majority of newly diagnosed children with T1D in our Danish cohort carry autoantibodies directed against the new autoantigen ZnT8. We have also replicated the strong correlation between autoantibodies specificity for the ZnT8 Arg325Trp isoforms and the rs13266634 genotype, which has been reported by others [10, 11]. Furthermore, we have investigated the relationship between ZnT8A and already established autoantibodies and could replicate the high overlap and the correlation between ZnT8A and IA-2A, previously reported [10, 12, 29, 30].

The ZnT8 Arg325Trp antibody specificity determined by the rs13266624 genotype is not absolute. However, in this study a larger portion of the ZnT8W only responders carry the CT genotype (49%) than has been reported in Caucasians. The study by Wenzlau et al. [10], reported a frequency of 28% for the CT genotype in the ZnT8W only group. A part of the discrepancy could be the higher minor allele frequency in our study material (0.34 vs. 0.27) which results in higher frequency of heterozygotes in our population (46.2% vs. 36.2%). In the group that reacted against both ZnT8 variants (ZnT8R+W), other epitopes most likely determine the ZnT8R response in TT homozygous children and ZnT8W response in CC homozygous children.

Previous studies have reported an increasing prevalence of ZnT8A with increasing age in children followed before disease onset [4, 12], as well as with age at diagnosis [4, 10] which is in concordance with our data. However, the prevalence pattern seems to be highly dependent on which age at diagnosis interval that is studied. Significantly higher prevalence and titers for ZnT8A were reported in a cohort diagnosed between 2-17 years of age compared to a diagnosis between 15-34 years of age [30]. After disease onset the prevalence and titers of ZnT8A decline significantly [30]. A trend for increasing prevalence with increasing age at diagnosis for IA-2A and GADA has also been reported previously [10], but in this study we observe significant correlations for GADA prevalence as well as titers. Our results indicate an age-dependent effect of autoantibody responses in T1D and support the measurement of ZnT8A, together with GADA and IA-2A, to be useful as a prognostic marker especially in individuals with an age at diagnosis above 5 years.

We and others have been unable to demonstrate a genetic association between rs13266634 and the risk of T1D [18-21]. However, ZnT8A positive children that carry the homozygous genotypes CC and TT progress faster to T1D than those carrying the CT genotype [12], highlighting that the additional risk stratified by ZnT8A can be improved further by rs13266634 genotyping in prospective studies. In a German study the rs13266634 C allele was associated with an age at diagnosis below 5 years [19]. It was proposed that this association could be coupled to a genetic susceptibility to β-cell dysfunction leading to an early manifestation of T1D in the presence of autoimmunity. Age at diagnosis was not affected by genotype in our cohort which is in accordance with other studies [10].

Given that rs13266634 changes an amino acid in ZnT8 which is important for insulin secretion and has documented effects on type 2 diabetes related traits [13-17], we hypothesised that the SNP could have an effect on β-cell function and the rate of β-cell destruction in T1D. In the absence of stimulated C-peptide measurements, we used IDAA1c as a proxy with values taken less than 1 year after diagnosis. The rs13266634 genotype had no effect on mean IDAA1c, however a non-significant trend towards a lower IDAA1c, and hence a better metabolic control, was found in carriers of the TT genotype. The lack of statistical significance in the analysis warrants a more thorough analysis in a larger study material, ideally using repeated stimulated C-peptid measurements. The lack of a correlation between ZnT8A and IDAA1c could indicate that a possible association between rs13266634 genotype and improved metabolic control might be independent of autoimmunity. However, it fits poorly with the fact that homozygote carriers that are positive for ZnT8A have an increased risk of progressing to T1D [12]. Any proven correlation between genotype and metabolic control, would offer a possibility for targeted interventions strategies but elucidation of these mechanism requires further studies.

The association between ZnT8R positivity and the *HLA-DQB1*0302* genotypes might depend on the correlation between ZnT8A and IA-2A, which is strongly associated with the **0302* allele, although this does not explain the lack of association for ZnT8W positivity with *HLA-DQB1*. However only speculative, the higher frequency of *DQB1*0302* genotypes in ZnT8R positive children could indicate an interaction between the binding specificity of the *DQB1*0302* molecule and the Arg325 epitope in ZnT8. The modest p-values are suggestive and warrant an investigation of this finding in a larger material.

In conclusion, our study has further characterised the relationship between autoantibody specificity for the ZnT8 Arg325Trp epitopes and rs13266634 genotypes in relation to established autoantibodies, age at diagnosis, measures of β-cell function and *HLA-DQB1* genotypes in T1D. Our results confirm that ZnT8 is a major target for autoantibodies at disease onset in our Danish T1D cohort of children and adolescents. The rs13266634 variant in *SLC30A8* controls the autoantibody specificity for the ZnT8R and ZnT8W isoforms, respectively. However, specific genotypes could not be correlated to an effect on residual β-cell function nor age at diagnosis. Children with an age at onset below 5 years had significantly lower prevalence and titers of ZnT8A, although the effect was largely mediated by the ZnT8R variant when analysed separately. The ZnT8R variant was associated with the high risk *HLA-DQB1*0302* genotypes whereas ZnT8W was independent of *HLA-DQB1.* These results could help to guide studies focusing on the mechanisms by which the immune system recognise and lose self-tolerance for the ZnT8 Arg325Trp isoforms in T1D.

ACKNOWLEDGEMENT

This study was funded by research grants from EFSD/JDRF/Novo Nordisk, the Juvenile Diabetes Research Foundation (33-2008-391), Novo Nordisk A/S, the EU 7th Framework Programme DIAPREPP (grant agreement 202013), the Swedish Research Council (2007-2266) and the Swedish Diabetes Association. The Danish Study Group of Childhood Diabetes (DSBD) biobank is funded by grants from the Danish Medical Research Council (271-07-0657) and the Danish Diabetes Association. We greatly acknowledge Rikke Bonne and Zohreh Sattari for skilful laboratory assistance.

REFERENCES

[1] Kulmala P, Savola K, Petersen JS, Vahasalo P, Karjalainen J, Lopponen T, Dyrberg T, Akerblom HK, Knip M. Prediction of insulin-dependent diabetes mellitus in siblings of children with diabetes. A population-based study. The Childhood Diabetes in Finland Study Group. J Clin Invest 1998; 101: 327-336.

[2] The Environmental Determinants of Diabetes in the Young (TEDDY) study: study design. Pediatr Diabetes 2007; 8: 286-298.

[3] Krischer JP, Cuthbertson DD, Yu L, Orban T, Maclaren N, Jackson R, Winter WE, Schatz DA, Palmer JP, Eisenbarth GS. Screening strategies for the identification of multiple antibody-positive relatives of individuals with type 1 diabetes. J Clin Endocrinol Metab 2003; 88: 103-108.

[4] Wenzlau JM, Juhl K, Yu L, Moua O, Sarkar SA, Gottlieb P, Rewers M, Eisenbarth GS, Jensen J, Davidson HW, Hutton JC. The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. Proc Natl Acad Sci U S A 2007; 104: 17040-17045.

[5] Chimienti F, Devergnas S, Favier A, Seve M. Identification and cloning of a beta-cell-specific zinc transporter, ZnT-8, localized into insulin secretory granules. Diabetes 2004; 53: 2330-2337.

[6] Chimienti F, Devergnas S, Pattou F, Schuit F, Garcia-Cuenca R, Vandewalle B, Kerr-Conte J, Van Lommel L, Grunwald D, Favier A, Seve M. In vivo expression and functional characterization of the zinc transporter ZnT8 in glucose-induced insulin secretion. J Cell Sci 2006; 119: 4199-4206.

[7] Sladek R, Rocheleau G, Rung J, Dina C, Shen L, Serre D, Boutin P, Vincent D, Belisle A, Hadjadj S, Balkau B, Heude B, Charpentier G, Hudson TJ, Montpetit A, Pshezhetsky AV, Prentki M, Posner BI, Balding DJ, Meyre D, Polychronakos C, Froguel P. A genome-wide association study identifies novel risk loci for type 2 diabetes. Nature 2007; 445: 881-885.

[8] Zeggini E, Weedon MN, Lindgren CM, Frayling TM, Elliott KS, Lango H, Timpson NJ, Perry JR, Rayner NW, Freathy RM, Barrett JC, Shields B, Morris AP, Ellard S, Groves CJ, Harries LW,

Marchini JL, Owen KR, Knight B, Cardon LR, Walker M, Hitman GA, Morris AD, Doney AS, McCarthy MI, Hattersley AT. Replication of genome-wide association signals in UK samples reveals risk loci for type 2 diabetes. Science 2007; 316: 1336-1341.

[9] Scott LJ, Mohlke KL, Bonnycastle LL, Willer CJ, Li Y, Duren WL, Erdos MR, Stringham HM, Chines PS, Jackson AU, Prokunina-Olsson L, Ding CJ, Swift AJ, Narisu N, Hu T, Pruim R, Xiao R, Li XY, Conneely KN, Riebow NL, Sprau AG, Tong M, White PP, Hetrick KN, Barnhart MW, Bark CW, Goldstein JL, Watkins L, Xiang F, Saramies J, Buchanan TA, Watanabe RM, Valle TT, Kinnunen L, Abecasis GR, Pugh EW, Doheny KF, Bergman RN, Tuomilehto J, Collins FS, Boehnke M. A genome-wide association study of type 2 diabetes in Finns detects multiple susceptibility variants. Science 2007; 316: 1341-1345.

[10] Wenzlau JM, Liu Y, Yu L, Moua O, Fowler KT, Rangasamy S, Walters J, Eisenbarth GS, Davidson HW, Hutton JC. A common nonsynonymous single nucleotide polymorphism in the SLC30A8 gene determines ZnT8 autoantibody specificity in type 1 diabetes. Diabetes 2008; 57: 2693-2697.

[11] Kawasaki E, Uga M, Nakamura K, Kuriya G, Satoh T, Fujishima K, Ozaki M, Abiru N, Yamasaki H, Wenzlau JM, Davidson HW, Hutton JC, Eguchi K. Association between anti-ZnT8 autoantibody specificities and SLC30A8 Arg325Trp variant in Japanese patients with type 1 diabetes. Diabetologia 2008; 51: 2299-2302.

[12] Achenbach P, Lampasona V, Landherr U, Koczwara K, Krause S, Grallert H, Winkler C, Pfluger M, Illig T, Bonifacio E, Ziegler AG. Autoantibodies to zinc transporter 8 and SLC30A8 genotype stratify type 1 diabetes risk. Diabetologia 2009; 52: 1881-1888.

[13] Steinthorsdottir V, Thorleifsson G, Reynisdottir I, Benediktsson R, Jonsdottir T, Walters GB, Styrkarsdottir U, Gretarsdottir S, Emilsson V, Ghosh S, Baker A, Snorradottir S, Bjarnason H, Ng MC, Hansen T, Bagger Y, Wilensky RL, Reilly MP, Adeyemo A, Chen Y, Zhou J, Gudnason V, Chen G, Huang H, Lashley K, Doumatey A, So WY, Ma RC, Andersen G, Borch-Johnsen K, Jorgensen T, van Vliet-Ostaptchouk JV, Hofker MH, Wijmenga C, Christiansen C, Rader DJ, Rotimi C, Gurney M, Chan JC, Pedersen O, Sigurdsson G, Gulcher JR, Thorsteinsdottir U, Kong

A, Stefansson K. A variant in CDKAL1 influences insulin response and risk of type 2 diabetes. Nat Genet 2007; 39: 770-775.

[14] Staiger H, Machicao F, Stefan N, Tschritter O, Thamer C, Kantartzis K, Schafer SA, Kirchhoff K, Fritsche A, Haring HU. Polymorphisms within novel risk loci for type 2 diabetes determine beta-cell function. PLoS ONE 2007; 2: e832.

[15] Kirchhoff K, Machicao F, Haupt A, Schafer SA, Tschritter O, Staiger H, Stefan N, Haring HU, Fritsche A. Polymorphisms in the TCF7L2, CDKAL1 and SLC30A8 genes are associated with impaired proinsulin conversion. Diabetologia 2008; 51: 597-601.

[16] Boesgaard TW, Zilinskaite J, Vanttinen M, Laakso M, Jansson PA, Hammarstedt A, Smith U, Stefan N, Fritsche A, Haring H, Hribal M, Sesti G, Zobel DP, Pedersen O, Hansen T. The common SLC30A8 Arg325Trp variant is associated with reduced first-phase insulin release in 846 non-diabetic offspring of type 2 diabetes patients--the EUGENE2 study. Diabetologia 2008; 51: 816-820.

[17] Pare G, Chasman DI, Parker AN, Nathan DM, Miletich JP, Zee RY, Ridker PM. Novel association of HK1 with glycated hemoglobin in a non-diabetic population: a genome-wide evaluation of 14,618 participants in the Women's Genome Health Study. PLoS Genet 2008; 4: e1000312.

[18] Brorsson C, Bergholdt R, Sjogren M, Eising S, Sorensen KM, Hougaard DM, Orho-Melander M, Groop L, Pociot F. A non-synonymous variant in SLC30A8 is not associated with type 1 diabetes in the Danish population. Mol Genet Metab 2008; 94: 386-388.

[19] Gohlke H, Ferrari U, Koczwara K, Bonifacio E, Illig T, Ziegler AG. SLC30A8 (ZnT8) Polymorphism is Associated with Young Age at Type 1 Diabetes Onset. Rev Diabet Stud 2008; 5: 25-27.

[20] Qu HQ, Grant SF, Bradfield JP, Kim C, Frackelton E, Hakonarson H, Polychronakos C. Association analysis of type 2 diabetes Loci in type 1 diabetes. Diabetes 2008; 57: 1983-1986.

[21] Raj SM, Howson JM, Walker NM, Cooper JD, Smyth DJ, Field SF, Stevens HE, Todd JA. No association of multiple type 2 diabetes loci with type 1 diabetes. Diabetologia 2009; 52: 2109-2116.

[22] Egefjord L, Jensen JL, Bang-Berthelsen CH, Petersen AB, Smidt K, Schmitz O, Karlsen AE, Pociot F, Chimienti F, Rungby J, Magnusson NE. Zinc transporter gene expression is regulated by pro-inflammatory cytokines: a potential role for zinc transporters in beta-cell apoptosis? BMC Endocr Disord 2009; 9: 7.

[23] Hampe CS, Hammerle LP, Bekris L, Ortqvist E, Kockum I, Rolandsson O, Landin-Olsson M, Torn C, Persson B, Lernmark A. Recognition of glutamic acid decarboxylase (GAD) by autoantibodies from different GAD antibody-positive phenotypes. J Clin Endocrinol Metab 2000; 85: 4671-4679.

[24] Grubin CE, Daniels T, Toivola B, Landin-Olsson M, Hagopian WA, Li L, Karlsen AE, Boel E, Michelsen B, Lernmark A. A novel radioligand binding assay to determine diagnostic accuracy of isoform-specific glutamic acid decarboxylase antibodies in childhood IDDM. Diabetologia 1994; 37: 344-350.

[25] Sjoroos M, Iitia A, Ilonen J, Reijonen H, Lovgren T. Triple-label hybridization assay for type-1 diabetes-related HLA alleles. Biotechniques 1995; 18: 870-877.

[26] Mortensen HB, Hougaard P, Swift P, Hansen L, Holl RW, Hoey H, Bjoerndalen H, de Beaufort C, Chiarelli F, Danne T, Schoenle EJ, Aman J. New Definition for the Partial Remission Period in Children and Adolescents with T1D. Diabetes Care 2009.

[27] Nielsen LB, Ploug KB, Swift P, Orskov C, Jansen-Olesen I, Chiarelli F, Holst JJ, Hougaard P, Porksen S, Holl R, de Beaufort C, Gammeltoft S, Rorsman P, Mortensen HB, Hansen L. Co-localisation of the Kir6.2/SUR1 channel complex with glucagon-like peptide-1 and glucose-dependent insulinotrophic polypeptide expression in human ileal cells and implications for glycaemic control in new onset type 1 diabetes. Eur J Endocrinol 2007; 156: 663-671.

[28] Graham J, Hagopian WA, Kockum I, Li LS, Sanjeevi CB, Lowe RM, Schaefer JB, Zarghami M, Day HL, Landin-Olsson M, Palmer JP, Janer-Villanueva M, Hood L, Sundkvist G, Lernmark A,

Breslow N, Dahlquist G, Blohme G. Genetic effects on age-dependent onset and islet cell autoantibody markers in type 1 diabetes. Diabetes 2002; 51: 1346-1355.

[29] De Grijse J, Asanghanwa M, Nouthe B, Albrecher N, Goubert P, Vermeulen I, Van Der Meeren S, Decochez K, Weets I, Keymeulen B, Lampasona V, Wenzlau J, Hutton JC, Pipeleers D, Gorus FK. Predictive power of screening for antibodies against insulinoma-associated protein 2 beta (IA-2beta) and zinc transporter-8 to select first-degree relatives of type 1 diabetic patients with risk of rapid progression to clinical onset of the disease: implications for prevention trials. Diabetologia; 53: 517-524.

[30] Vaziri-Sani F, Oak S, Radtke J, Lernmark A, Lynch K, Agardh CD, Cilio CM, Lethagen AL, Ortqvist E, Landin-Olsson M, Torn C, Hampe CS. ZnT8 autoantibody titers in type 1 diabetes patients decline rapidly after clinical onset. Autoimmunity. [Early Online]

FIGURE LEGENDS

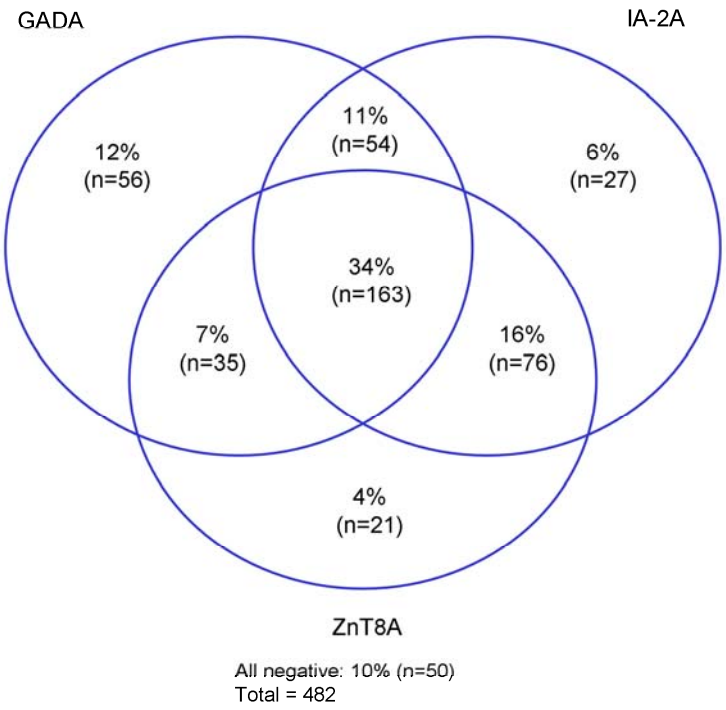

Figure 1. Overlap between the prevalence of ZnT8A, GADA and IA-2A in diabetic probands.

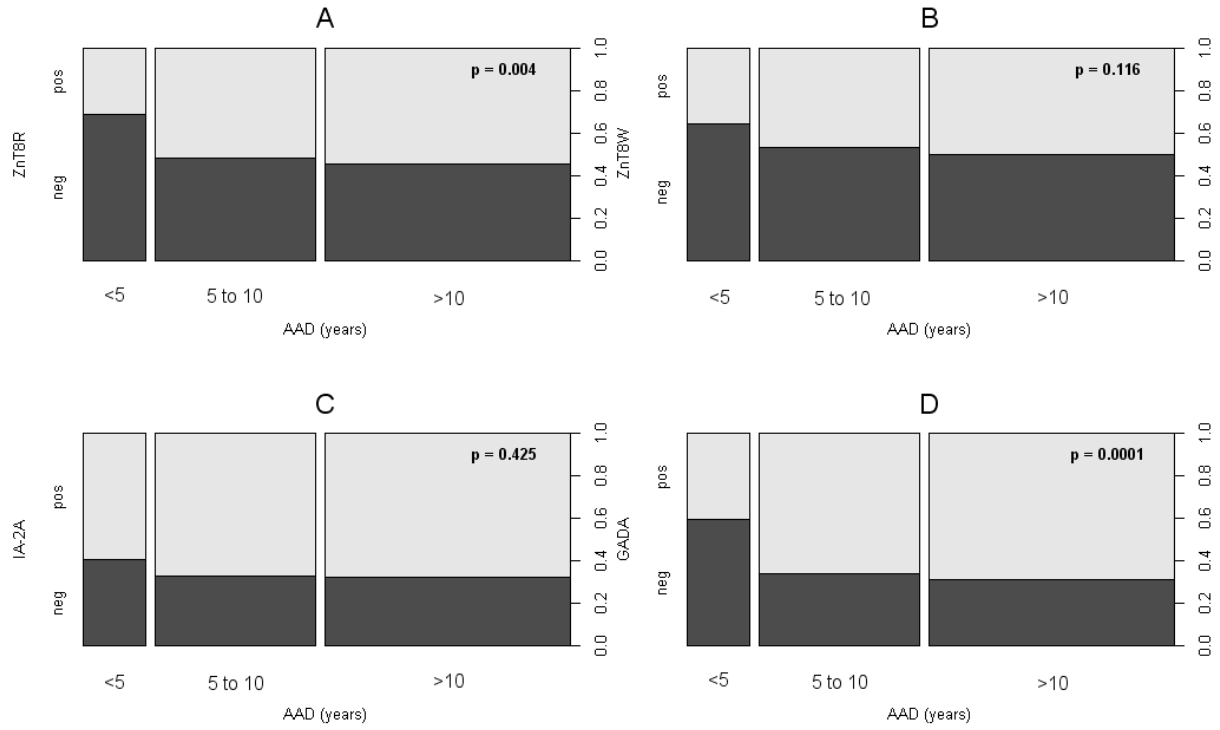

Figure 2. Autoantibody status correlated to age at diagnosis (AAD). **A**) ZnT8R **B**) ZnT8W **C**) GADA and **D**) IA-2A. Differences between the age groups were evaluated with a χ^2-test.

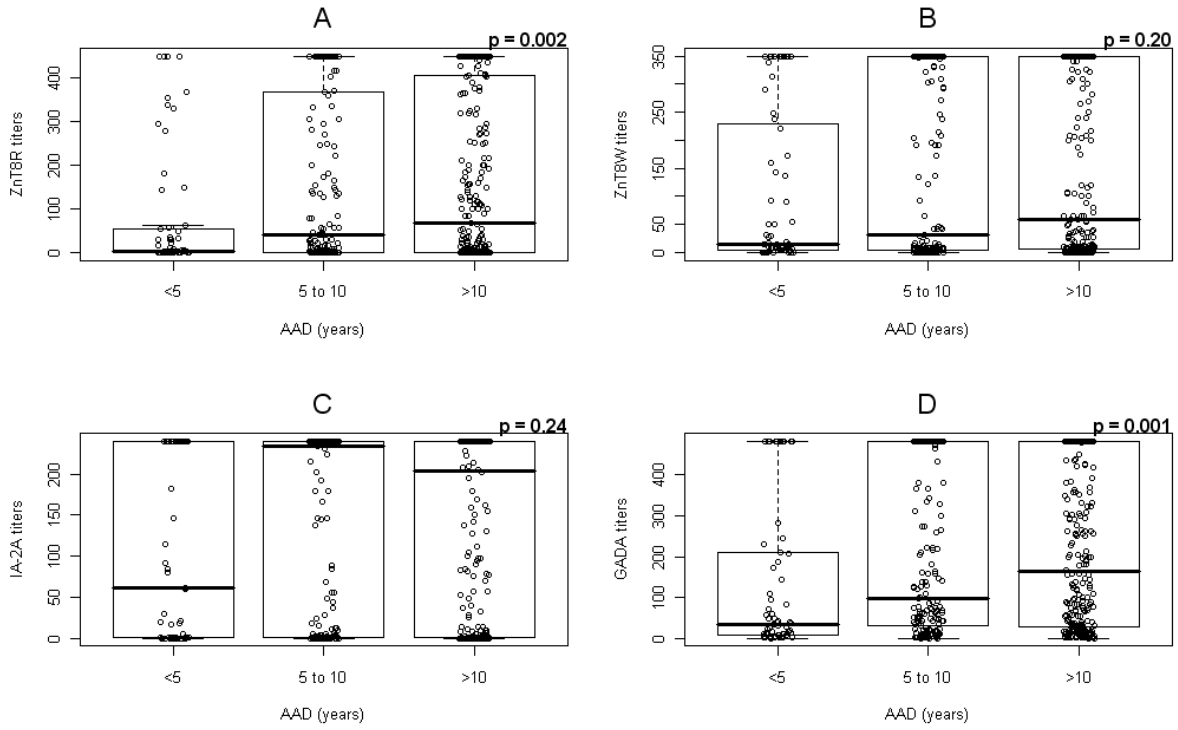

Figure 3. Autoantibody titers correlated to age at diagnosis (AAD). **A**) ZnT8R **B**) ZnT8W **C**) GADA and **D**) IA-2A. Differences between the age groups were evaluated with a Kruskal-Wallis rank sum test.

TABLES

Table 1. Distribution of autoantibody positive probands and siblings for ZnT8A, IA-2A and GADA.

	Probands (N=482)	**Siblings (N=478)**	**P-value**
ZnT8A n (%)	295 (61.2)	9 (1.9)	<2.2e-16
IA-2A N (%)	320 (66.4)	10 (2.1)	<2.2e-16
GADA n (%)	308 (63.9)	23 (4.8)	<2.2e-16

Antibody positivity was defined as having a value >30 U/ml for the Arg epitope and > 58 U/ml for the Trp epitope and > 6 U/ml and > 50 U/ml for IA2A and GADA, respectively.

Table 2. rs13266634 genotype distribution for autoantibody positive probands.

rs13266634 genotype	CC (N=198)	CT (N=210)	TT (N=50)	Total (N=458)	P-value
ZnT8R n (%)	131 (66.2)	90 (42.9)	11 (22.0)	232	1.542e-09
ZnT8W n (%)	74 (37.4)	107 (51.0)	36 (72.0)	217	2.513e-05
IA-2A n (%)	135 (68.2)	142 (67.6)	33 (66.0)	310	0.9571
GADA n (%)	137 (69.2)	126 (60.0)	30 (60.0)	293	0.1274
rs13266634 genotype	**CC (N=59)**	**CT (N=33)**	**TT (N=25)**	**Total (N=117)**	**P-value**
ZnT8R-only n (%)	58 (98.3)	8 (24.2)	0 (0.0)	66	
ZnT8W-only n (%)	1 (1.7)	25 (75.8)	25 (100.0)	51	<2.2e-16
ZnT8R+W n (%)	73 (0)	82 (49.0)	11 (6.6)	166	

Table 3. Test of the correlation between positivity for IA-2A, GADA and ZnT8A in probands.

	ZnT8A-pos (N=295)	ZnT8A-neg (N=187)	P-value
IA-2A-pos n (%)	239 (81.0)	81 (43.3)	<2.2e-16
GADA-pos n (%)	198 (67.1)	110 (58.8)	0.065

Antibody positivity was defined as having a value >30 U/ml for the Arg epitope and > 58 U/ml for the Trp epitope and > 6 U/ml and > 50 U/ml for IA2A and GADA, respectively.

Table 4. Age at diagnosis (AAD) correlated to autoantibody positivity in probands.

	AAD < 5 (N=64)	AAD 5-10 (N=165)	AAD > 10 (N=252)	Total (N=481, 1 NA)	P-value
ZnT8A n (%)	29 (45.3)	103 (62.4)	163 (64.7)	295	0.017
IA2A n (%)	38 (59.4)	111 (67.3)	171 (67.9)	320	0.425
GADA n (%)	26 (40.6)	109 (66.1)	173 (68.7)	308	0.0001

Table 5. *HLA-DQB1* genotype distribution in autoantibody positive probands.

HLA genotype	DQB1*02 (N=67)	DQB1*0302 (N=292)	Moderate and Protective (N=87)	Total (N=446)	P-value
ZnT8R n (%)	28 (41.8)	159 (54.5)	34 (39.1)	221	0.01628
ZnT8W n (%)	28 (41.8)	139 (47.6)	44 (50.6)	211	0.5489
IA2A n (%)	29 (43.3)	217 (74.3)	51 (58.6)	297	1.613e-06
GADA n (%)	54 (80.6)	178 (61.0)	49 (56.3)	281	0.003912
HLA genotype	**DQB1*02 (N=17)**	**DQB1*0302 (N=76)**	**Moderate and Protective (N=23)**	**Total (N=116)**	**P-value**
ZnT8R-only	8 (47.1)	48 (63.2)	7 (30.4)	63	
ZnT8W-only	9 (52.9)	28 (36.8)	16 (69.6)	53	0.018

DQB1*02: 02_X/604; **DQB1*0302**: 302_2/604/X;

Moderate and protective: 301_2/X; 304_2/X; 603_2/302/X; 604_301/X; X_X; 602_**;

Table 6. Mean IDAA1c divided by rs13266634 genotypes in probands with values taken within 1 year of diagnosis. Mean age at diagnosis and diabetes duration within groups are displayed for comparison.

	rs13266634			
	CC	**CT**	**TT**	**P-value**
N (%)	104 (43.0)	116 (47.9)	22 (9.1)	
Mean	9.91	9.70	9.52	0.48
IDAA1c (95% C.I.)	(9.56-10.27)	(9.36-10.03)	(8.75-10.29)	
Median	9.87	9.52	9.13	
St. Dev.	1.83	1.89	1.67	
Mean diabetes duration	0.58	0.53	0.50	0.27
(range, years)	(0.08-1.0)	(0.02-0.98)	(0.07-0.97)	
Mean AAD	8.7	9.4	10.7	0.049
(range, years)	(1-18)	(1-15)	(2-17)	

Differences between groups are evaluated with a Kruskal-Wallis rank sum test.

IDAA1c=Insulin-dose adjusted HbA1c, AAD=age at diagnosis