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Running title

Zinc Transporter 8 autoantibodies/SLC30A8 in T1D

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ZnT8 autoantibodies and their association with *SLC30A8* and *HLA-DQ* genes differ between immigrant and Swedish patients with newly diagnosed type 1 diabetes in the Better Diabetes Diagnosis (BDD) study.

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Abstract

Objective

We examined whether zinc transporter-8 autoantibodies (ZnT8A) differed between immigrant and Swedish T1D patients due to differences in genetic polymorphisms of *SLC30A8*, *HLA-DQ,* or both.

Research design and Methods

Newly diagnosed (May 2005-January 2011) autoimmune (≥1 islet autoantibody) T1D patients (n=2964, <18y, 55% male) were ascertained in the BDD study. Three subgroups identified: Swedes (n=2160, 73%), immigrants (non-Swedes; n=212, 7%) and Swedish mixed-origin (n=592, 20%).

Results

Non-Swedes had less of the autoantibody ZnT8-WA (38%) than Swedes (50%; $p=0.001$) consistent with a lower frequency in the non-Swedes (37%) of the *SLC30A8* CT+TT (RW+WW) genotypes than in the Swedes (54%; $p=0.0005$). ZnT8-RA (57% and 58%, respectively) didn't differ despite a higher frequency of CC (RR) genotypes in non-Swedes (63%) than Swedes (46%; $p<0.0005$). We tested if this inconsistency was due to HLA-DQ as 2/X (2/2; 2/y; y is anything but 2 or 8), which was a major genotype in non-Swedes (40%) compared to Swedes (14%; $p=0.0005$). In the non-Swedes only, 2/X (2/2 or 2/y) were negatively associated with ZnT8-WA ($p=0.008$), ZnT8-QA ($p=0.03$) but not ZnT8-RA ($p=0.26$). Molecular simulation showed non-binding of the relevant ZnT8R peptide to DQ2 explaining in part the possible lack of tolerance to ZnT8-R. Compared to all other DQ genotypes, non-Swedes with DQ2/y ($p<0.002$), but not 2/2 ($p=0.88$) had an increased frequency of *SLC30A8*-CC consistent with ZnT8-RA. In Swedes, however, DQ8/y carriers were predominately CT+TT ($p=0.03$) consistent with ZnT8-WA.

Conclusion

At diagnosis in non-Swedes, the presence of ZnT8-RA rather than ZnT8-WA was likely due to an additive effect between *HLA-DQ2* and the *SLC30A8* CC(RR) genotypes.

Keywords

SLC30A8, ZnT8 autoantibodies, HLA-DQ genes, type 1 diabetes, migration.

Abbreviations

BDD: Better Diabetes Diagnosis Study

GAD65A: Glutamic Acid Decarboxylase Autoantibodies

IA-2A: Islet Antigen-2 Autoantibodies

IAA: Insulin Autoantibodies

T1D: Type 1 Diabetes

ZnT8A: Zinc Transporter 8 autoantibodies

ZnT8-RA: Zinc Transporter 8 autoantibodies: Arginine variant

ZnT8-WA: Zinc Transporter 8 autoantibodies: Tryptophan variant

ZnT8-QA: Zinc Transporter 8 autoantibodies: Glutamine variant

Introduction

More than 90% of childhood type 1 diabetes (T1D) in Caucasian populations are classified as autoimmune diabetes, which is associated with Human Leukocyte Antigen (HLA) class II genes (1). The autoimmune response involves production of islet autoantibodies, their type and number assists in prediction (2, 3) and classification (4) of T1D.

Recently, the zinc transporter 8 (ZnT8) was described as a target of autoimmunity in childhood T1D (5, 6) and adult-onset autoimmune diabetes (7). The ZnT8 is a 41 kDa membrane protein of beta-cell secretory granules and a member of the zinc transporter (ZNT)/SLC30 subfamily of the cation diffusion facilitator family (8). ZnT8 is thought to play an essential role in insulin crystallization and secretion through permitting cellular efflux of zinc (8, 9). Two SNPs of the ZnT8 genes, *SLC30A8,* determine single amino acid (aa) variation at position 325 of the cytosolic segments of ZnT8: 1) rs13266634, which codes for either arginine (CGG) or tryptophan (TGG), 2) rs16889462, which codes for glutamine (CAG) (5, 10). The single amino acid polymorphism at position 325 of ZnT8 identifies three antigenic variants: Arginine (ZnT8-R), Tryptophan (ZnT8-W) or Glutamine (ZnT8-Q).

Autoantibodies against the ZnT8 (ZnT8A) were found in 63% of T1D patients, 2% of health controls and 3% of T2D patients (6). ZnT8A were also detected among 26% of T1D patients who were negative for other islet autoantibodies (6). Therefore it has been suggested that adding ZnT8A would detect more than 95% of T1D patients (11). Furthermore, ZnT8A were detected in 81% of children who progressed to T1D in BABYDIAB study (12) indicating their importance in prediction of autoimmune childhood diabetes. This progression to diabetes was found to be associated with CC genotype of *SLC30A8*, which was also associated with the younger (<5 years) newly diagnosed T1D patients (13). However, Genome Wide Association Studies (GWAS) did not yet confirm the association between *SLC30A8* and T1D (14). On the other hand, the *SLC30A8* is confirmed to be associated with T2D (15) where the risk C allele was found to confer 14% and 16% increased risk for T2D in European-ancestry populations and Asians respectively (16). Furthermore, 46% of European non-diabetic offspring of T2D patients are homozygous for CC genotype of the *SLC30A8* and are prone to diabetes (17). In T1D patients, the C allele (R) of *SLC30A8* genotype was previously shown to be associated with higher stimulated C-peptide levels (18) during the first year following

diagnosis. This suggests that the two main variants (RR) and (WW) of ZnT8A may be associated with or reflect different clinical outcomes in pediatric T1D patients.

Recent data suggest that DQ molecules can modulate autoimmune response through differential bindings to islet autoantigen fragments (19). These data showed that the binding of the insulin antigenic peptide to HLA-DQ alleles may induce regulatory or proinflammatory responses of T cells depending on which DQ molecule involved in this binding. On the other hand, *in vitro* studies showed that the peptide pools containing the whole 369 aa ZnT8 sequence are targeted by autoreactive T cells especially the DR3-DQ2 and DR4-DQ8 carriers (20) indicating their importance in ZnT8 presentation.

In Sweden, 12–15% of children <18 years have non-Swedish backgrounds. Immigrant children are at an increased risk of T1D in Sweden, where they present different HLA-DQ and islet autoantibody associations from native Swedish patients (21). We therefore studied the three ZnT8A variants (ZnT8-RA, ZnT8-WA and ZnT8-QA) and their *SLC30A8* and HLA-DQ associations among immigrants (non-Swedish) and Swedish patients with newly diagnosed T1D. We also investigated the T1D-susceptible HLA-DQ peptide-binding motifs within the whole 369 aa ZnT8 sequence focusing on the region around amino acid polymorphism at position 325.

Aim and hypothesis

The aim was to determine whether the frequencies of ZnT8A variants differ between non-Swedish and Swedish patients with childhood autoimmune T1D (≥1 autoantibody) and whether such differences are due to differences in the genetic polymorphisms of *SLC30A8* or HLA-DQ genotypes or both. We also determined the specific binding epitopes of HLA-DQ alleles throughout the 369 aa ZnT8 protein.

We hypothesized that non-Swedish patients, compared to Swedish patients, have different frequencies of ZnT8A related to differences in the *SLC30A8* genotypes but also depends on differences in the HLA-DQ genotypes, which determine specific affinities towards ZnT8 autoantigen.

Research design and methods

Study design

Participants were recruited from the national Better Diabetes Diagnosis (BDD) study in Sweden. The BDD study design was previously described (21). The World Health Organization criteria for diagnosis and classification of T1D were used to determine clinical diagnosis (22, 23), however, we included only patients with autoimmune T1D who were positive for at least one out of six islet autoantibodies. Dried blood spots (DBS) for HLA-DQ typing, blood for *SLC30A8* genotyping and serum samples for islet autoantibodies (GAD65A, IA-2A, IAA, ZnT8-RA, ZnT8-WA and ZnT8-QA) were used.

The "ethnic" origin was defined by *country of birth* of parents and grandparents and was obtained from a questionnaire.

The Karolinska Institute Ethics Board approved the BDD study (2004/1:9).

Study population

A total of 3686 newly diagnosed patients (<18 years) with childhood diabetes in the BDD study during May 2005 to January 2011 were recruited. We identified a total of 2964 patients with autoimmune T1D for whom autoimmune status and country of birth were known. Three subgroups were identified based on the origin of all parents and grandparents. These groups were Swedes (2160, 73%), non-Swedes (212, 7%), and Swedish-mixed origins (592, 20%) (Figure 1). Non-Swedes were defined as patients whose parents and grandparents were *all* born outside Sweden and Swedes were patients whose parents and grandparents were *all* born in Sweden. Although non-Swedes were not homogenous; they shared geographical/cultural backgrounds: Middle East and North Africa (MENA including Somalia: 58%), South-East Europe (mainly former Yugoslavia: 24%), Western-Northern Europe (9%), and other mixed origins (9%). The basis for this grouping was previously described (21).

The majority (86%) of autoimmune T1D patients were diagnosed before 15 years of age and almost 18% diagnosed before the age of 5. Males (1879, 55%) had slightly higher (10.1, SD=4.45) mean age of diagnosis than females (9.4, SD=4.24) (Table 1).

Islet autoantibody analysis

Glutamic Acid Decarboxylase autoantibodies (GAD65A) and Islet antigen-2 autoantibodies (IA-2A).

Recombinant GAD65 and IA-2 were labelled with ^{35}S-methionine (GE Healthcare Life Sciences, Amersham, UK) by *in vitro* coupled transcription and translation in TNT SP6 coupled reticulocyte lysate system (Promega, Southampton, UK) as described (24). Full length cDNA coding for human GAD65 in the pTNT vector (Promega) (pThGAD65) or the intracellular domain (amino acids 603-980) of IA-2 in the pSP64 Poly(A) vector (Promega) (IA-2ic) were used (25). GAD65A and IA-2A were analyzed in a radioligand binding assay (24) in samples eluted from dried blood spots. Discs at the size of 6 mm in diameter were punched (Wallac DBS puncher, PerkinElmer Life and Analytical Sciences, Brussels, Belgium) from DBS on filter paper (grade 2992 filters; Schleicher and Schuell, Dassel, Germany). The DBS discs were incubated over night at +4°C in 80 μL Tris-Buffered Saline with Tween 20 (TBST) with shaking to elute antibodies. In the autoantibody assays, 30 μL DBS eluate was incubated with 24 000 cpm of ^{35}S-labelled GAD65 or IA-2 in TBST in a final reaction volume of 60 μL. The samples were transferred to filtration plates (Millipore, Solna, Sweden) and free ^{35}S-labelled GAD65 or IA-2 separated from antibody bound with Protein A-Sepharose (Zymed Laboratories Inc, San Francisco, CA, USA). After washing with TBST, the plates were allowed to dry. Supermix scintillation cocktail (Perkin Elmer) was added and the radioactivity of antibody bound ^{35}S-labelled GAD65 or IA-2 counted in a Wallac Microbeta Trilux (Perkin Elmer) beta counter. GAD65A and IA-2A levels were expressed as units per mL (U/mL) derived from the WHO standard 97/550 (26). Samples were considered positive if GAD65A levels were above 50 U/mL and IA-2A levels above 10 U/mL. The intra-assay coefficient of variation for duplicates in the GAD65A assay was 7% and in the IA-2A 11%. In the DASP 2009 workshop our laboratory was among the top ranking laboratories for GAD65A in workshop sensitivity (68%) and specificity (99%) and the top ranking laboratory for IA-2A in workshop sensitivity (60%) and specificity (99%).

Insulin Autoantibodies (IAA)

Non-competitive method.

Insulin auto-antibodies (IAA) were analyzed in serum samples. A non-competitive method was performed to detect IAA using radio-labeled ^{125}I–insulin (27). Serum

samples (7 μl) were added to duplicate wells of a 96-well microplate and 36 μl of [125]I - insulin with an activity of 60 000 cpm/well was added then incubated on shaker at +4°C for 48 h. Protein A-Sepharose (PAS) in a 40% slurry (50 μl) was added to a filter plate and washed three times with 200 μl Tris buffer using a Micro-Plate Strip Washer. After incubation of the serum with ^{125}I–insulin, 25 μl was transferred to the filter plate with PAS and the plate was incubated on a shaker at +4 °C for 1.5 h. The plates were washed 10 times with 200 μl Tris buffer. Supermix scintillation solution (50 μl) was added to the wells after the plate had dried for 15 minutes. The radioactivity was measured in a beta counter (Wallac Micro Beta Trilux, Perkin Elmer).

Competitive method

Positive samples for IAA were further analyzed using a competitive method. Serum samples (7 μl) were added to four wells on a 96-well plate. To these wells, 36 μl of ^{125}I-insulin with an activity of 60 000 cpm/well was added, with 0,072 IU (or 2 IU/mL) unlabeled insulin (Actrapid, Novo Nordisk) added in the last two wells. The plates were incubated and examined under similar conditions as in the non-competitive method. IAA levels were calculated as relative units (RU) and were related to positive controls. Positivity for IAA was set to 1.0 RU.

Zinc transporter 8 autoantibodies (ZnT8A)

Serum samples were analyzed for ZnT8A: Arginine (ZnT8-RA), Tryptophan (ZnT8-WA) and Glutamine (ZnT8-QA) using the radioligand binding assay previously described (28). Briefly, the c-terminal constructs of ZnT8 were prepared using a Phusion™ site-directed mutagenesis kit (Finnzymes Oy, Espoo, Finland). The ^{35}S–methionine labeled antigens were incubated over night at 4°C with duplicate serum samples followed by precipitation of immune complexes with protein A Sepharose (Amersham Biosciences, Uppsala, Sweden). The antibody-bound radioactivity was counted in a β-counter (1450 MicroBeta TriLux Microplate Scintillation-Luminescence Counter) and concentration of antibodies were estimated from a known standard curve and analyzed in GraphPad PRISM 4.0 software.

SLC30A8 **genotyping.**

DNA was isolated from whole blood of the newly diagnosed patients using the plasmid Max isolation kit (Qiagen, City State) according to the manufacturer's instructions. The *SLC30A8* genotype at amino acid position 325 was detected with the SNP rs13266634 with use of matrix-assisted laser spectrophotometry as previously described (29, 30). The *SLC30A8* genotypes were grouped into "risk" CC and "lower-risk" CT+TT genotypes.

HLA typing

The HLA-DQA1 and B1 genotypes were typed using sequence-specific oligonucleotide probes on DBS used directly for PCR amplification of DQA1 and DQB1 alleles as described (31) using a DELFIA Hybridization assay (Perkin Elmer, Boston, MA). The first set of probes defines the presence of HLA-DQB1*02, 0302, 0301, 0602, 0603 and 0604. The second set of probes defines the presence of additional DQB1 alleles. HLA-DQA1 probes defines the DQA1*0201, 03 and 05 alleles. The HLA-DQ genotypes were grouped based on the presence of DQ8 (A1*03:01-B1*03:02) and DQ2 (A1*05:01-B1*02:01) haplotypes into four groups 1) DQ2/8 (patients who have both DQ2 and DQ8), 2) DQ8/X (patients who have either homozygous DQ8/8 or heterozygous DQ8/y: where y is any other haplotype except DQ8 or DQ2), 3) DQ2/X (patients have either homozygous DQ2/2 or heterozygous DQ2/y: where y is any other haplotype except DQ2 or DQ8) and 4) DQX/X (patients have neither DQ8 nor DQ2). DQ6.4 was recognized as a risk allele within nine high risk genotypes in the BDD cohort and therefore was further analyzed as DQ6.4 allele regardless of previous grouping where it could be grouped under DQ2/X, 8/X or X/X.

Epitope scanning and molecular simulation of HLA-DQ—peptide epitope complexes

The ZnT8 molecule (all 3 variants) was scanned for epitopes to HLA-DQ2, HLA-DQ8, HLA-DQ2trans (A1*03:01/B1*02:01) and HLA-DQ8trans (A1*05:01/B1*03:02), using the established motifs of these alleles (19, 32-36). It was shown that there were two sets of epitopes fulfilling the motifs for alleles HLA-DQ2 and –DQ8 in the polymorphic region around residue 325, i.e. peptide 319-327 **V**AT**AA**S(*RWQ*)D**S** and peptide 321-329 **T**AA**S**(*RWQ*)**DS**Q**V** (core nonamers, polymorphic residues in italics in parenthesis, anchors in bold). In the first epitope the p7R variant is a non-binder to HLA-DQ2.

Molecular simulation of the complexes of HLA-DQ alleles and peptides were performed as previously described (19, 36). Briefly, the complexes of HLA-DQ2—gliadin peptide, and HLA-DQ8—insulin B11-23 peptide were used as base molecules for the respective HLA-DQ2 and –DQ8 complexes; in the case of complexes with the DQ2trans and DQ8trans alleles models of the trans alleles were built by superposition of the two DQcis structures and selection of two combinations. In all cases the peptide coordinates used were those of Insulin B11-23 as the gliadin peptide contained four prolines within the core nonamer sequence, an unlikely circumstance for type 1 diabetes autoantigens. The most suitable rotamers were chosen in the case of the antigenic peptide residues and the energy minimization process consisted of 1000 steps of steepest gradient and another 1000 steps of the conjugate gradient using the program Discover of Accelrys (San Diego, CA, USA) on an Octane or a Fuel instrument of Silicon Graphics (Fremont, CA, USA). Figures of modeled structures were made using the DSViewer Pro and of Accelrys.

Statistical analysis

SPSS 18® statistical package (SPSS Inc. Chicago, Illinois, US) was used for statistical analysis. Pearson Chi square test of independence (and Yates' correction for continuity value when applied) was used to assess relationships between ZnT8A, *SLC30A8* and HLA. Logistic regression models -tested at 95% confidence interval level- were used to assess whether any of the *SLC30A8* and HLA-DQ genotypes were independently associated with ZnT8A and country of birth.

Results

ZnT8WRQA

Among the autoantibody-positive T1D patients (n=2964), a total of 2021 (68%) were positive for at least one ZnT8A variant, 1226 (41%) had multiple (≥2/3) ZnT8A and 880 (30%) were positive for all three variants. All three variants showed gradual increase in frequency with age at diagnosis. They were least common those below 5 years and the highest frequency occurred in the 10-15 years age group in both non-Swedes and Swedes. In general, Swedish patients had frequently two or more ZnT8A (69%) than non-Swedes (62%) (p=0.02) (Table 1). This difference was mainly due to ZnT8-WA being more common in Swedes (50%) than among non-Swedes (38%; p=0.001) (Figure 2).

SLC30A8 genotypes and ZnT8A

The *SLC30A8* (rs13266634) genotypes distribution differed between non-Swedes and Swedes. The T2D risk CC genotype was more common among non-Swedes (63%) than Swedes (46%), (p<0.0005) (Table 2). When comparing CC to CT+TT, the CC genotype was more frequent in non-Swedes at younger age of diagnosis (<5 years; OR=2.5 (95%CI=1.1-5.6), p=0.02) and (5-10; OR=2.87 (95%CI=1.6-5.1), p=0.0002) but there was no age variation in Swedes for these genotypes.

The CC genotype was associated with ZnT8-RA in both non-Swedes (p=0.002) and Swedes (p<0.0005). Similarly CT+TT genotype was associated with ZnT8-WA in non-Swedes (p=0.004) and Swedes (p<0.0005).

ZnT8A and HLA DQ genotypes

In non-Swedes HLA-DQ 2/X (2/2; 2/y; y is any haplotype but 2 or 8) was both the main genotype (40%) and more common than in Swedes (14%; p=0.0005).

In non-Swedes, DQ2/X were negatively associated with multiple (≥2/3) ZnT8A (p=0.02), ZnT8-WA (p=0.008) and ZnT8-QA (p=0.03) but not ZnT8-RA (p=0.26).

DQ2/8, which was more frequent in Swedes (37%) than in non-Swedes (18%; (p=0.005)) could not explain the low frequency of ZnT8-WA in the non-Swedes (p=0.38). DQ8/X (8/8 or 8/y; y is any haplotype but 2 or 8), however, was associated with multiple ZnT8A (p=0.016) as well as with all three variants, ZnT8-RA (p=0.04), ZnT8-WA (p=0.03) and ZnT8-QA (p=0.01). DQ6.4 (44% of the non-Swedes were DQ2/6.4)

showed positive association with ZnT8-WA only (*p*=0.04), although this association was no longer significant when corrected for continuity (*p*=0.7).

In Swedes, all three ZnT8A variants were associated with DQ2/8 (*p*<0.0005) and DQ8/X (*p*<0.0005). However, DQX/X (non-DQ2/non-DQ8) were also associated with ZnT8-RA (*p*=0.04) and ZnT8-QA (*p*=0.01) but not with ZnT8-WA while DQ2/X did not show any association. DQ6.4 (59% of all DQ6.4 in the Swedes was present in the DQ8/6.4 genotype) showed positive association with all three ZnT8A variants (*p*<0.0005).

SLC30A8 and HLA DQ genotypes

As expected from the autoantibody results, non-Swedes with DQ2/X (DQ2/2 or 2/y; y is any DQ haplotype but 2 or 8)) had a higher frequency of CC (75%) than CT+TT (25%; *p*=0.009) genotypes. In Swedes, however, 57% of the DQ8/X (DQ8/8 or 8/y; y is any DQ haplotype but 2 or 8) carriers the CT+TT genotype (*p*=0.02). These findings were explained by the heterozygous carriers of DQ2/y in non-Swedes (*p*=0.002) and carriers of DQ8/y in Swedes (*p*=0.03) but not by the homozygous DQ2/2 and DQ8/8 carriers, respectively.

Logistic regression models showed that having a CC genotype of the *SLC30A8* (OR=1.95 (95%CI=1.4-2.7), *p*=0.0005) and DQ2/X (OR=1.6 (95%CI=1.0-2.5), *p*=0.04) were associated with non-Swedish origins, while having DQ2/8 (OR=0.36 (95%CI=0.22-0.61), *p*<0.0005) and DQ8/X (OR=0.34 (95%CI=0.21-0.56), *p*<0.0005), were negatively associated with non-Swedish origin.

HLA-DQ-ZnT8 peptide bindings

The analysis for ZnT8 motifs for DQ8, DQ2 and DQ6.4 showed that DQ2 epitopes bindings throughout the whole ZnT8 369 amino acid peptide (including 325R/W/Q) were more abundant than DQ8 and DQ6 epitopes (Table 4). There were 7 very good and many other intermediate epitopes for DQ2 compared to 2 strong and several intermediate epitopes for each of DQ8 and DQ6.4. However, none of the strong epitopes involved the polymorphic 325RWQ position within 319-329R/W/Q but there were 6 intermediate binders for DQ8, 3 for DQ6.4 and only 2 combinations for DQ2 (Table 4). The epitopes 319-327 including Tryptophan (W) at position 7: VATAAS*W*DS and 319-327 including Glutamine (Q) at position: VATAAS*Q*DS but not Arginine (R) may also bind

weakly to DQ2 indicating that it selectively binds to epitopes of W and Q but not the R variant (Figure 4). Of the strong binding epitopes, the nearest to the 325R/W/Q position were (344-351: MHSLTIQM) for DQ8 and (352-360: ESPVDQDPD) for DQ2. The molecular simulation photo of HLA-DQ2 in complex with ZnT8 peptide 319-327p7Q is shown in Figure 4. Of the five different anchors (p1, p4, p6, p7, p9) and the one shelf (p3) we note that p4A is a weak anchor, while all others are good to very good anchors.

Discussion

In this study we demonstrated that immigrant patients (non-Swedes) who were born in Sweden to non-Swedish parents and grandparents develop ZnT8A with genetic associations different from that in Swedish patients. Non-Swedes tended to have a higher frequency of the T2D risk CC genotype of the *SLC30A8* genotype than Swedes (*p*=0.0005) and as expected, ZnT8-RA rather than the ZnT8-WA. Our data also showed that the DQ2 haplotype, which had more epitope binding sites to ZnT8 than the DQ8 haplotype. The ZnT8W and Q but not R-containing peptides was found to bind weakly to the DQB1*0604 allele- containing DQ heterodimer, which may explain the differences in ZnT8A frequencies between non-Swedes and Swedes. Finally, among the non-Swedes, but not the Swedes, the CC genotype was associated with younger age of diagnosis.

The importance of ZnT8/*SLC30A8* in diabetes is increasing since they appear to have dual roles; ZnT8 is an autoantigen in T1D (37) and the C allele of *SLC30A8* is associated with T2D but not T1D (14, 38). Polymorphism of two SNPs of *SLC30A8* (rs13266634 and rs16889462) replacing a single amino acid at position 325 of the cytosolic segment of ZnT8 identifies 3 antigenic targets of islet autoimmunity (10, 37). The high frequency (>63%) of ZnT8A in T1D compared to less than 3% of T2D patients (5), highlights the importance of these autoantibodies as disease markers in T1D although their exact role in pathogenesis is yet to be fully understood. It is an enigma why the two SLC30A8 SNPs are associated with T2D but not T1D despite the SNPs are associated with the risk of developing T1D with ZnT8 autoantibodies.

The observed differences in the ZnT8A between non-Swedes and Swedes were related to differences in their genetic heritage. This difference may not be solely explained by the predominance of *SLC30A8* CC genotype in non-Swedes. Data from the international HapMap project (39) showed that the CC genotype is more frequent in non-Caucasian African populations than Europeans and Asians. In our cohort, almost 60% of non-Swedes originate from Middle Eastern and African countries, which may explain why the CC genotype is more prevalent in these patients. The C allele was previously found to be associated with younger age of onset of T1D patients in the BABYDIAB study (13). Interestingly enough, we detected this age variation only among non-Swedes but not among the Swedes.

More interestingly, the DQ2/X (2/2 or 2/y), which was the main HLA-DQ genotype in non-Swedes, showed negative association with ZnT8-WA (*p*=0.008) but not with ZnT8-

RA and was abundant in the CC ($p<0.009$) carriers only. Of interest, this finding was evident only with the carriers of the heterozygous DQ2/y ($p<0.002$) but not the homozygous DQ2/2 individuals and was not influenced by the DQ2/8 genotype. Therefore, we proposed that the lower frequency of ZnT8-WA in non-Swedes may be explained both by the low frequency of the CT+TT (RW+WW), and the high frequency of DQ2/X. This phenomenon was not observed in the Swedes. Previous studies showed that DQ8 was associated mainly with ZnT8-RA (40) and DQ6.4 were associated with ZnT8-RA and ZnT8-WA (41). Therefore, the DQ6.4 association with ZnT8A, especially ZnT8-WA, may be independent from DQ2 as seen in non-Swedes but potentiated by DQ8 as seen in Swedes although the DQ6.4 association in the non-Swedes were limited by the number of patients.

The stronger DQ2 associations in non-Swedes may reflect different affinity of DQ2 for the respective antigenic peptides around the polymorphic 325RWQ position. Our ZnT8 epitope binding data showed that the overall number of epitopes binding to DQ2 exceeds the number of epitopes binding to DQ8 and also to DQ6.4 although DQ8 had more intermediate bindings around the polymorphic 325RWQ position. The higher binding affinity of DQ2 may in fact be related to its highly versatile pockets (19, 32, 33) that can bind several aliphatic, aromatic and acidic-polar residues (abundant in ZnT8). These properties are different from DQ8, which has a narrower preference spectrum of residues for its pockets. The epitope binding analysis close to the 325RWQ position, showed that DQ2 had weak binding affinity towards the tryptophan- (W) and glutamine- (Q) but no binding to the arginine- (R) containing epitope (nonapeptide 319-327): VATAAS*W*DS, VATAAS*Q*DS and VATAAS*R*DS (Figure 4). These data may suggest that DQ2 preferably binds, albeit weakly, to W- and Q-containing peptides thereby reducing the chance of an immune response against these variants through central tolerance mechanisms in the thymus and perhaps in the periphery. By contrast, DQ8 and 6.4 carriers are less likely to promote tolerance mechanisms against ZnT8 variants. Taken together, the lower frequency ZnT8-WA and ZnT8-QA may be due to the moderating effects of DQ2 through tolerance.

ZnT8 is proposed to regulate insulin secretion from beta cells through cumulating zinc in beta-cell granules (42). Deletion of *slc30a8* in rodents caused moderate disturbances in insulin secretion (43, 44). In humans, healthy children (9-11 and 14-16 years) in the European Youth Heart Study showed successive increase in fasting glucose with

increase in number of SNPs of diabetes-related risk alleles (45). In these subjects, the *SLC30A8* (rs13266634) was the only T2D variant associated with high fasting glucose. Additionally, the risk C allele of *SLC30A8* (rs13266634) was found to be associated with beta-cell dysfunction but not insulin resistance (46). On the other hand, zinc transporting system in beta cells, in particular ZnT8 was shown to be sensitive to cytokine (IL-1β) induced apoptosis (47). Furthermore, recent findings suggest that patients with recent onset T1D (<6 months) expressed significantly higher autoreactive T cells against ZnT8 compared to controls (20). Upon processing of proinsulin to insulin, zinc in the beta-cell granules may associate with insulin (48). The above studies suggest that ZnT8/*SLC30A8* may predispose to T1D through two mechanisms: 1) autoimmune and inflammatory mechanisms involved in islet autoimmunity and 2) compromising beta-cell function. Therefore, our findings may suggest that the role of ZnT8 in T1D, especially in non-European descent populations is determined mostly by HLA-DQ alleles but also by *SLC30A8* genotypes. The antigen-presentation of DQ-ZnT8 complexes may induce tolerance or promote autoimmune response against the ZnT8-containing beta cells, depending on a variety of factors, such as strength of peptide binding and cognate TCR binding, as well as respective level of expression of HLA-DQ and autoantigen in central APC, peripheral APC, or both. . There has been no reported expression of ZnT8 in the thymus or peripheral APC, as has been the case for the acetylcholine receptor protein (autoantigen for myasthenia gravis), another integral membrane protein, albeit at the cell surface and not in a secretion granule such as ZnT8 (49, 50). Further studies on larger and more homogenous groups of T1D patients from non-European Caucasian populations may provide insights on the contribution of *SLC30A8* and other T2D genes in the risk for T1D.

Conclusion

Non-Swedish patients develop T1D predominately with ZnT8-RA rather than ZnT8-WA at diagnosis likely due to immune tolerance to the (W)-containing peptide brought about by HLA-DQ2 rather than DQ8. In these patients, the CC genotype *SLC30A8* may provide an additive effect to the HLA-DQ risk and increase predisposition to T1D. These findings also suggest that HLA-DQ molecules may modulate autoimmune response in T1D depending on their peptide binding affinities.

Appendix

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Conflict of interests

The authors declare no conflicts of interests.

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Tables and Figures

Table 1. General characteristics of non-Swedes and Swedes including autoimmune and genetic data.

	Origin (country of birth)	
	Non-Swedes	**Swedes**
n (%)	212 (7)	2160 (73)
Mean age (SD)	9.4 (4.1)	9.8 (4.4)
Males n (%)	105 (50)	1187 (55)
Autoimmunity n (%)		
IA-2A	139 (66)	1728 (80) *
GAD65A	150 (71)	1306 (61) *
IAA	64 (30)	749 (35)
ZnT8A (≥2/3)	131 (62)	1499 (69)
ZnT8-RA	120 (57)	1244 (58)
ZnT8-WA	81 (38)	1083 (50) *
ZnT8-QA	64 (30)	729 (34)
Multiple Aab (≥2/6)	165 (78)	1814 (84)
HLA-DQ n (%)	**212 (100)**	**2160 (100)**
2/8	37 (18)	660 (31) §
8/X†	45 (21)	897 (42) §
2/X‡	83 (40)	309 (14) §
X/X	44 (21)	269 (13) §

† DQ8/X (03:02/X) includes DQ8/8 and DQ8 with any other combination except DQ2.
‡ DQ2/X (05 02/X) includes DQ2/2 and DQ2 with any other combination except DQ8.

* ZnT8-WA and IA2A were less frequent in non-Swedes than Swedes ($p=0.001$, $p<0.0005$) respectively while GAD65A were more frequent in non-Swedes than Swedes ($p=0.003$).
§ Non-Swedes predominately had DQ2 ($p<0.0005$) compared to Swedes who had DQ8 ($p<0.0005$) and DQ2/8 ($p<0.0005$).

Table 2 The SLC30A8 (SNP rs13266634) genotypes in Non-Swedes and Swedes.*

Age group (y)	Non-Swedes n (%)			Swedes n (%)		
	CC	CT+TT	Total	CC	CT+TT	Total
<5	20 (67)	10 (33)	30 (100)	123 (44)	154 (56)	277 (100)
5-<10	46 (72)	18 (28)	64 (100)	250 (47)	281 (53)	531 (100)
10-<15	32 (53)	28 (47)	60 (100)	324 (47)	360 (53)	684 (100)
15-18	7 (58)	5 (42)	12 (100)	93 (42)	130 (58)	223 (100)

* The CC genotype predominated the non-Swedes while the CT+TT genotypes (*p*<0.0005) predominated the Swedes (*p*<0.0005). Unlike Swedes, the CC compared to CT+TT in non-Swedes was more frequent in younger patients (<5; OR=2.5 (95%CI=1.1-5.6), *p*=0.02) and (5-10; OR=2.87 (95%CI=1.6-5.1), *p*=0.0002).

Table 3. The *SLC30A8* genotypes in relation to HLA-DQ genotypes. *

HLA DQ genotypes	*SLC30A8* genotypes† n (%)					
	Non-Swedes			Swedes		
	CC	CT+TT	*P-value*	CC	CT+TT	*p-value*
All	104 (63)	62 (37)		787 (46)	926 (54)	
2/8	18 (60)	12 (40)	0.68	262 (50)	261 (50)	0.026‡
2/2	15 (60)	10 (40)	0.89	27 (46)	31 (54)	0.94
2/y§	34 (85)	6 (15)	**0.002**	93 (46)	108 (54)	0.95
8/8	4 (40)	6 (60)	-	94 (44)	120 (56)	0.51
8/y§	14 (56)	13 (44)	0.18	208 (42)	289 (58)	**0.03**
X/X	19 (56)	15 (44)	0.32	103 (47)	117 (53)	0.81

* The predominate CC genotype among non-Swedes was detected in association with HLA-DQ2/y (*p*=0.002) but not DQ2/2 while the CT+TT genotypes were more frequent in Swedes with HLA-DQ8/y (*p*=0.03) but not DQ8/8.

† Results for *SLC30A8* were not available for all cases.

‡ This *p* value reflects differences in non-DQ2/8 carriers.

§ y in DQ2/*y* is any haplotype other than DQ8 and in DQ8/*y* is any haplotype other than DQ2.

Table 4. ZnT8 epitopes restricted to T1D susceptible HLA-DQ haplotypes

Haplotype	Sequence*	Residue numbers	Comments on binding to HLA- DQ heterodimers*
HLA-DQ2	YA*F*T**LES**V**E	18-26	Excellent!
	EE*L*E**SGG**MY	43-51	GG may be a problem making the peptide too flexible
	EK*G*A**NEY**AY	61-69	p4A weak, other anchors very good
	ER*L***LYPD**Y**Q	164-172	good
	FV*H*A**LGD**LF	218-226	p4A weak, other anchors very good
	ES*P***VDQDP**D	352-360,	p6Q weak, other anchors very good
	VD*Q***DPDCL**F	355-363	very good
	VA*T*A**AS***W*DS	319-327W	P4A weak, other anchors good
	VA*T*A**AS***Q*DS	319-327Q	P4A weak, other anchors good
	VA*T*A**AS***R*DS	319-327R	Non-binder
	TA*AS***R***DS**QV	321-329R	P1**T** medium strength
	TA*AS***W***DS**QV	321-329W	P1**T** medium strength
	TA*AS***Q***DS**QV	321-329Q	P1**T** medium strength
HLA-DQ8	IC**F**IF**MI**AE	80-88	Very good
	MHS**LTIQ**ME	344-352	Very good
	VATA**AS***R*DS	319-327R	P7R acceptable, p9S weak
	VATA**AS***W*DS	319-327W	P7W acceptable, p9S weak
	VATA**AS***Q*DS	319-327Q	P7Q acceptable, p9S weak
	TAA*S***RDS**QV	321-329R	P1**T** medium strength, p9V weak
	TAA*S***WDS**QV	321-329W	P1**T** medium strength, p9V weak
	TAA*S***QDS**QV	321-329Q	P1**T** medium strength, p9V weak
HLA-DQ6.4	FI*F***SILVL**A	253-261	Very good
	IL*S*A**HVAT**A	314-322	Very good
	VA*T***AAS***R*DS	319-327R	Non-binder
	VA*T***AAS***W*DS	319-327W	Non-binder
	VA*T***AAS***Q*DS	319-327Q	Non-binder

	TA_ASR_**DS**Q**V**	321-329R	P1**T** weak, other anchors good to very good
	TA_ASW_**DS**Q**V**	321-329W	P1**T** weak, other anchors good to very good
	TA_ASQ_**DS**Q**V**	321-329Q	P1**T** weak, other anchors good to very good

*Only the well-binding epitopes are listed for the entire molecule. There is however a complete analysis of all possible epitopes around the 325W/Q/R polymorphic residue, shown in italics wherever appropriate. Only core nonamers of epitopes are shown with anchors p1, p4, p6, p7 and p9 in bold. Total numbers of the non-shown weak and intermediate epitopes are: 43 for HLA-DQ2, 14 for HLA-DQ8, and 15 for HLA-DQ604.

**HLA-DQ2 and –DQ604 have a p3 shelf, indicated by italicized residue.

Legends to figure

Figure 1.

Classification of patients according to country of birth of parents and grandparents (origin). A total of 2964 patients with autoimmune (≥1 autoantibody positive) T1D were included in this analysis. Non-Swedes (immigrants) accounted for 7% of patients, Swedes accounted for 73% while 20% had mixed Swedish/non-Swedish origins.

Figure 2. Venn diagram:

Panel A shows the frequencies and co-detection (% positive) of ZnT8A variants in non-Swedes and Swedes.

Panel B: 4 ellipses Venn diagram: ZnT8A (≥1 ZnT8 autoantibodies) were detected in 4.7% of non-Swedes and 3.4% of Swedes who were negative for conventional autoantibodies. Unlike Swedes, non-Swedes develop ZnT8A more frequently with GAD65A rather than IA-2A.

Figure 3. Co-detection of *SLC30A8* with HLA-DQ genotypes. In non-Swedes, DQ2/X was detected more frequently with CC ($p<0.009$). In Swedes, DQ8/X was detected more frequently with CT+TT ($p=0.02$).

Figure 4. ZnT8 epitops in complex with HLA-DQ alleles.

Panel A. TCR view of the modelled structure of the T1D susceptible HLA-DQ8 allele (A1*03:01-B1*03:02), in complex with The ZnT8 peptide 319-327, AH**V**AT**AASR**D**S**QV (anchors in bold, polymorphic residue in italics). The ZnT8 peptide is shown in Van der Waals solid surface form (atom color code: carbon, green; oxygen, red; nitrogen, blue; hydrogen, white; sulphur, yellow), while the α1β1 domain of the HLA-DQ molecule is shown in Van der Waals surface form with atom charges (positive, blue; negative, red; neutral, grey, and with appropriate scales of grey for situations in between). The polymorphic residue 325Arg occupies pocket 7, for which it is eminently suited in this allele.

Panel B.

TCR view of the modeled structure of the T1D susceptible HLA-DQ2 allele (A1*05:01-B1*02:01), in complex with The ZnT8 peptide 319-327, AH**VAT*AASW*DS**QV (anchors in bold, polymorphic residue in italics). Color conventions as in Figure 1. The polymorphic residue 325Trp occupies pocket 7, for which it is suited in this allele; HLA-DQ2 cannot tolerate Arginine in any of its pockets.

Panel C.

TCR view of the modeled structure of the T1D susceptible HLA-DQ2 allele (A1*05:01-B1*02:01), in complex with The ZnT8 peptide 319-327, AH**VAT*AASQ*DS**QV (anchors in bold, polymorphic residue in italics). Color conventions as in Figure 1. The polymorphic residue 325Gln occupies pocket 7, for which it is well-suited in this allele. Note that there are slight rearrangements both of peptide residues as well as HLA-DQ residues because of the p7Trp→Gln substitution, around the site of the substitution.
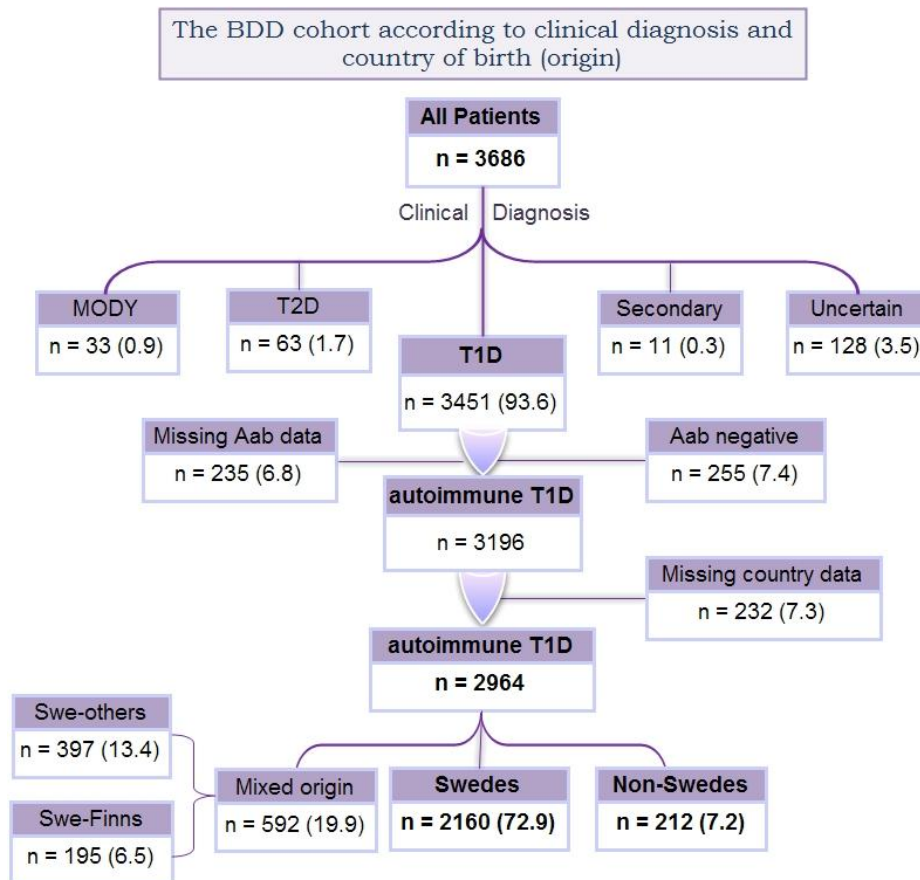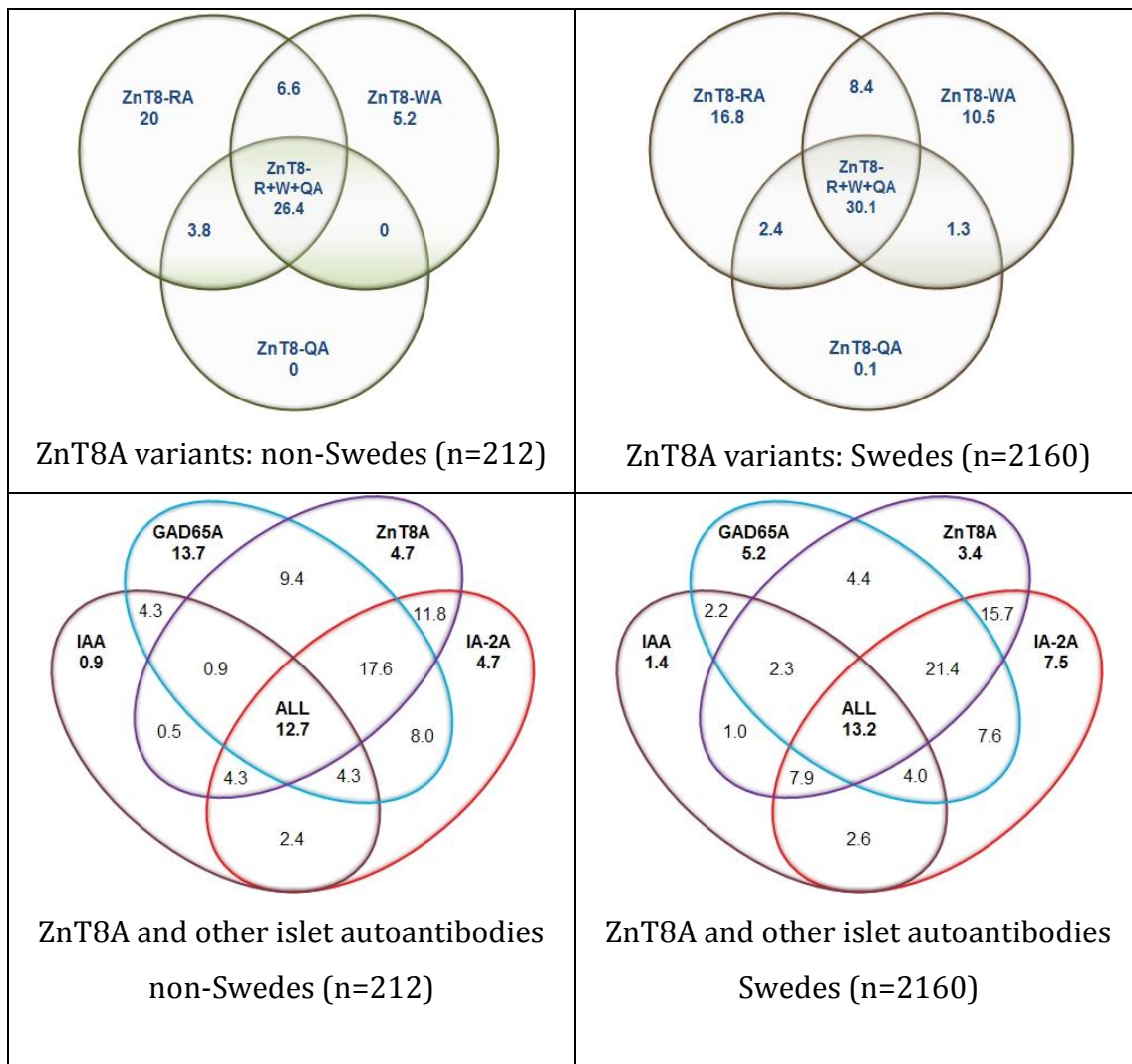
Figures

Figure 1.

Figure 2.

ZnT8A variants: non-Swedes (n=212)

ZnT8A variants: Swedes (n=2160)

ZnT8A and other islet autoantibodies
non-Swedes (n=212)

ZnT8A and other islet autoantibodies
Swedes (n=2160)

Figure 3

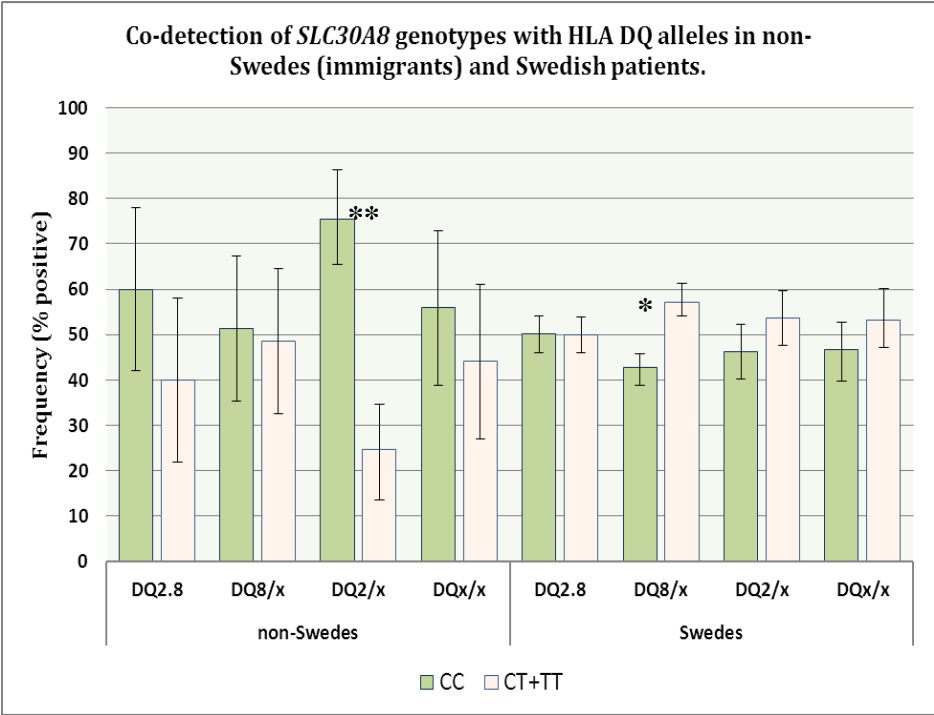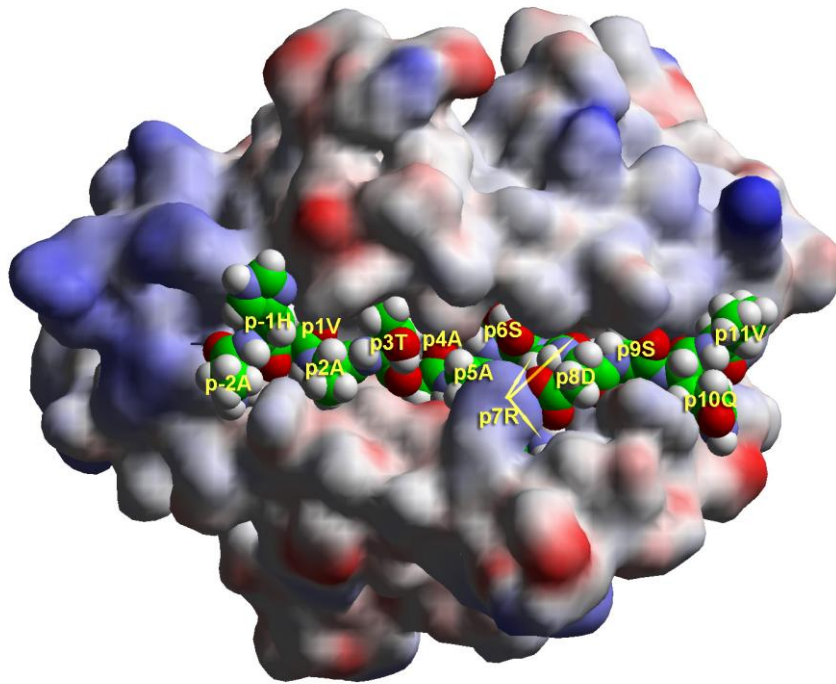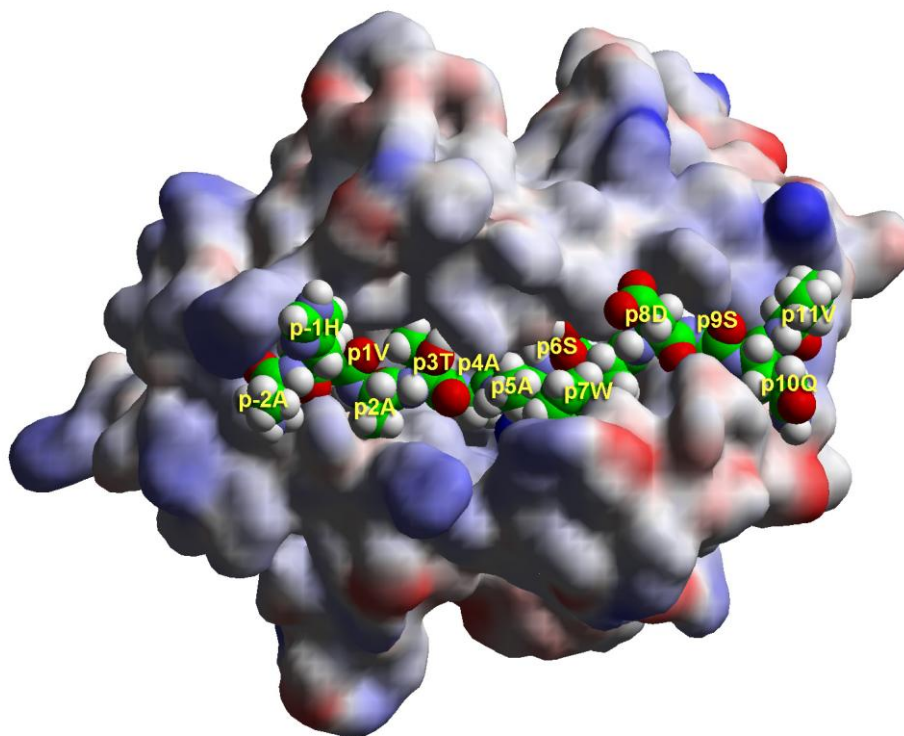Co-detection of *SLC30A8* genotypes with HLA DQ alleles in non-Swedes (immigrants) and Swedish patients.

Figure 4

Panel A

Panel B

Panel C