Aspects of ZnT8 autoimmunity in childhood type 1 diabetes

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Aspects of ZnT8 autoimmunity in childhood type 1 diabetes

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ASPECTS OF ZNT8 AUTOIMMUNITY IN CHILDHOOD TYPE 1 DIABETES

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Doctoral Dissertation

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# Table of Content

1. Abstract .................................................. 5
2. List of publications .................................... 7
3. Abbreviations ............................................. 9
4. Introduction ............................................. 11
   History .................................................. 11
   Clinical presentation and definition of diabetes 13
   Incidence and prevalence ............................. 14
   Pathogenesis ........................................... 15
      HLA .................................................. 18
      Environment ........................................ 20
      Islet autoantibodies ................................ 22
         \textit{ICA} ........................................ 25
         \textit{GADA} ....................................... 27
         \textit{IA-2A} ...................................... 28
         \textit{IAA} ......................................... 29
         \textit{ZnT8A} ...................................... 30
         \textit{VAMP2 and NPY} ......................... 35
      HbA1c ................................................. 35
      C-peptide ........................................... 35
      OGTT ............................................... 36
      IvGTT ............................................... 37
5. Aims and hypothesis

6. Subjects and Study Design

7. Methods

Autoantibody analysis
  ICA
  GADA
  IA-2A
  IAA
  ZnT8A

HLA
BMI
Plasma glucose
HbA1c
C-peptide and insulin
OGTT
IvGTT
Impaired glucose metabolism
Sample repository
Statistics
Ethical Aspects

8. Results and Discussion

Ranking and frequency of autoantibodies
Number of autoantibodies
Autoantibodies and their relation to age
Autoantibodies and gender
Can any of the autoantibody analysis replace another analysis?
HLA risk genotypes
HLA and autoantibodies 64
HLA and the relation to age 66
Islet autoantibody negative patients 67
ICA in islet autoantibody negative children and adolescents at type 1 diabetes diagnosis 68
Do patients positive for ICA display borderline levels of the other autoantibodies? 68
Do the ICA positive patients have type 1 diabetes 69
Can the Medizyme ICAscreen assay replace the ICA-IF assay? 69
Combinations of autoantibodies in multiple autoantibody positive non-diabetic children 70
BMI, fasting plasma glucose, HbA1c and fasting C-peptide 73
Glucose metabolism in non-diabetic children 73
Does the type, level or number of autoantibodies predict the glucose metabolism? 75
Do we need to analyze all three ZnT8A? 76

9. Conclusion and Future Perspectives 77
10. Sammanfattning på Svenska 81
11. Acknowledgements 85
12. References 87
13. Appendix: Papers I-IV 101
1. Abstract

The aim of this thesis was to investigate Zinc transporter 8 autoantibodies (ZnT8A), together with other known islet autoantibodies and human leukocyte antigen (HLA) in children with type 1 diabetes at clinical onset or children with high risk of progressing to type 1 diabetes. We found that:

1. ZnT8A were common in newly diagnosed type 1 diabetes children and adolescents and one of the three ZnT8A could be the only detectable islet autoantibody. Young children had as high diagnostic sensitivity for the ZnT8A as well as for multiple autoantibodies as older children. HLA DQA1-B1*0604 (DQ6.4) increased the risk for both Arginine and Tryptophan 325 Zinc transporter 8 autoantibodies (ZnT8RA and ZnT8WA, respectively) but not for Glutamine 325 Zinc transporter 8 autoantibodies (ZnT8QA). Agreement between autoantibody pairs was common for all combinations except insulin autoantibodies (IAA), but the different islet autoantibody analyses could not replace each other.

2. We confirmed that ZnT8A were independent of age in children and adolescents when diagnosed with type 1 diabetes. However, the contribution of ZnT8 autoimmunity to type 1 diabetes below the age of two was limited. ZnT8A increase the diagnostic sensitivity of islet autoantibodies for type 1 diabetes. Only 7% of the Swedish children were autoantibody negative. As many as 3.4% (108/3165) children had only ZnT8A at diagnosis. ZnT8QA was not found as a single islet autoantibody and the analysis of ZnT8QA at diagnosis may not be cost effective as a diagnostic marker. All variants of ZnT8A were positively associated with either HLA DQ6.4 or HLA DQA1*0301-DQB1*0302 (DQ8) but dominantly negative with HLA DQA1*0501-DQB1*0201 (DQ2). The association between DQ 6.4 and all three ZnT8A may be related to ZnT8 antigen-presentation by the DQ6.4 heterodimer.

3. A vast majority of children diagnosed with diabetes in Sweden had autoimmune type 1 diabetes as 91% were positive for at least one
islet autoantibody. Only 0.3% of type 1 diabetes patients had an isolated positivity for islet cell cytoplasm autoantibodies (ICA), but this suggests that another yet unidentified autoantigen or autoantigens may contribute to ICA. The commercial Enzyme-Linked Immunosorbent Assay (ELISA) Medizym ICAscreen was proper for screening of ICA positivity but positive samples need to be reanalyzed with a two-colour indirect immunofluorescence ICA assay (ICA-IF) to confirm ICA positivity.

4. Young healthy children with multiple autoantibodies may have impaired glucose metabolism. Children with autoantibodies to glutamic acid decarboxylase (GADA) and at least one other islet autoantibody and impaired glucose metabolism tended to be positive for ZnT8QA, HLA DQA1*0501-DQB1*0201 / DQA2*0301-DQB2*0302 (DQ2/8), or both. These results suggest that both immunogenetic and islet autoimmunity markers rather than number of islet autoantibodies may be of importance when inviting and selecting young children with islet autoantibodies to secondary prevention trials. Evaluation of baseline metabolic control is critical and should be taken into account before randomization.

Our data support the view that it is important to analyze all known islet autoantibodies in attempts to predict type 1 diabetes and to assist in classification of diabetes, although the analysis of GADA and insulinoma-associated protein 2 autoantibodies (IA-2A) may suffice initially as these two autoantibodies are most cost efficient and identify most type 1 diabetes patients. Different HLA might explain the different antigen-presentations in patients. Evaluation of baseline metabolic control is important in healthy children before randomization, since glucose metabolism may differ between healthy children with islet autoantibodies and the affected glucose metabolism could affect the outcome of studies.
2. List of Publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:


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3. Abbreviations

A  Autoantibody/Autoantibodies
ADA  American Diabetes Association
AUC  Area under the curve
BDD  Better Diabetes Diagnosis
BMI  Body mass index
CI  Confidence interval
CTLA-4  Cytotoxic T-lymphocyte-associated protein 4/
        IDDM12
CV  Coefficient of variation
DASP  Diabetes Autoantibody Standardization Program
DBS  Dried blood spots
DiAPREV-IT  Diabetes Prevention -Immune Tolerance
DiPiS  Diabetes Prediction in Skåne study
DQ2  Haplotype HLA-DQA1*0501-DQB1*0201
DQ8  Haplotype HLA-DQA1*0301-DQB1*0302
DQ2/8  Genotype HLA-DQA1*0501-DQB1*0201/
        DQA2*0301-DQB2*0302
DQ6.4  Haplotype HLA-DQA1*X-DQB1*0604
ELISA  Enzyme-linked immunosorbent assay
FPIR  First phase insulin response
GADA  Autoantibodies to glutamic acid decarboxylase
GWAS  Genome-wide association studies
HLA  Human leukocyte antigen
IAA  Insulin autoantibodies
IA-2A  Insulinoma-associated protein 2 autoantibodies
ICA  Autoantibodies to islet cell cytoplasm
ICA-IF  Two-colour indirect immunofluorescence ICA assay
IDDM  Insulin-dependent diabetes mellitus genes
INS  Insulin gene / insulin-VNTR / IDDM2
IvGTT  Intravenous glucose tolerance test
JDF-U  Juvenile Diabetes Foundation Unit
MHC  Major histocompatibility complex
MODY  Maturity onset diabetes of the young
NPY  Neuropeptide Y
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>OGTT</td>
<td>Oral glucose tolerance test</td>
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<td>OR</td>
<td>Odds ratio</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>RBA</td>
<td>Radioligand binding assay</td>
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<tr>
<td>SNP</td>
<td>Single-nucleotide polymorphism</td>
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<td>SWEDIABKIDS</td>
<td>The Swedish childhood diabetes register</td>
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<td>TBST</td>
<td>Tris-Buffered Saline with Tween</td>
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<td>TEDDY</td>
<td>The Environmental Determinants of Diabetes in the Young study</td>
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<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
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<tr>
<td>VAMP2</td>
<td>Vesicle-associated membrane protein 2</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<td>ZIP</td>
<td>Zinc transporter(s) from the SLC39A gene family</td>
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<td>ZnT</td>
<td>Zinc transporter(s) from the SLC30A gene family</td>
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<tr>
<td>ZnT8A</td>
<td>Zinc transporter 8 autoantibodies to either one, two or all three amino acid variants at position 325</td>
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<tr>
<td>ZnT8RA</td>
<td>Arginine 325 Zinc transporter 8 autoantibodies</td>
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<td>ZnT8WA</td>
<td>Tryptophan 325 Zinc transporter 8 autoantibodies</td>
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<td>ZnT8QA</td>
<td>Glutamine 325 Zinc transporter 8 autoantibodies</td>
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4. Introduction

Type 1 diabetes is one of the most common chronic diseases in childhood and adolescence. The incidence is rapidly increasing worldwide and children tend to be younger at clinical onset. The young age affects both the child and the family, who needs to take meticulous control of the child in order to have a good metabolic control of the disease, so that the child can live a normal life and avoid future complications of the disease. At the same time the family needs to balance the control over the child so that he or she can grow up to be an independent individual with excellent knowledge of the disease and an enthusiasm for keeping a good metabolic control through life. The overall aim of this thesis was, firstly, to investigate autoimmune markers, especially the ZnT8A, together with HLA alleles, haplotypes and genotypes in children and adolescents with type 1 diabetes or high risk of developing type 1 diabetes. Another aim was to use the results for correct classification of diabetes and the final aim was to investigate to what extent these markers can predict differences in glucose metabolism in non diabetic children.

History

The earliest description of diabetes is found in the Egyptian Ebers papyrus from about 1500 B.C. About a thousand years later the first clinical diagnosis of diabetes was described by Susruta of India but it was not until 164 B.C. that the Greek Arataeus introduced the name ‘diabetes’. The origin of the term diabetes mellitus is both from Greek and Latin. The Greek root for diabetes means ‘siphon’, referring to polyuria and the Latin word mellitus means ‘honey-sweet’, referring to glucosuria. The diagnosis was made by people tasting the urine until the 11th century outside Europe. It was not until the 17th century that the sweet taste in the urine of people with diabetes was mentioned in the medical literature of Europe. In 1776 Dr Matthew Dobson demonstrated that boiling urine until evaporation left a substance with the sweetness of sugar, and the same crystalline
characteristics. Dr Dobson also discovered that sugar was present in the blood of diabetic patients [1-2].

Thomas Cawley discovered, in 1788, that insulin was produced and stored in the pancreas, when he noticed that the pancreas was shrivelled in a diabetic patient at autopsy. In 1869 Paul Langerhans discovered islet cells in the pancreas but he did not explain their function. Joseph von Mering and Oscar Minkowski discovered, in 1889, that removal of the pancreas in dogs resulted in the development of diabetes. Finally, in 1893, Gustav Laguesse suggested that the islet cells in the pancreas were involved in internal secretion helping sugar control and named them the islet of Langerhans after their discoverer. This was confirmed by Moses Barron who found, at autopsy, that the islets of Langerhans were damaged in a patient with diabetes. He concluded that this was the cause of diabetes and a possible treatment would be the substance from these cells. Sir Edward Albert Sharpey-Schafer named this substance ‘insulin’ in 1910. The Latin word insula means island.

Frederick Banting, Charles Best and John Macleod proved that insulin was the treatment for diabetes as they isolated and extracted insulin from the pancreas in healthy dogs in 1921, and injected it into dogs with diabetes lowering the blood sugar concentration, although the first dog died of insulin coma the next day. With the help of James Collip, Banting and Best purified insulin and in January 1922 treated Leonard Thompson, a 14-year-old boy with diabetes, with intramuscular insulin for the first time. The 1923 Nobel Prize in Physiology and Medicine was awarded to Banting and Macleod for this achievement. This insulin caused some local inflammation at the injection sites, but Collip helped with further purification and Thompson survived until the age of 27, when he died of pneumonia [1-2].

Type 1 diabetes had been fatal until 1922. People were treated with different diets both with high and low sugar and carbohydrate contents with a short survival of the patients [1]. The role of HLA genotypes in diabetes was first discovered in 1973 when a strong linkage of type 1 diabetes to the highly polymorphic HLA class II immune recognition molecules – DR and, later, DQ – located on chromosome 6p21 was detected [3-4]. G.F. Bottazzo discovered the first autoantibodies to pancreatic islet cells in 1974 and confirmed that type 1 diabetes was of autoimmune origin, which had been suspected for a while [5].
Clinical presentation and definition of diabetes

Typical symptoms of diabetes are polydipsia, polyuria, weight loss, fatigue and blurred vision. If diagnosis is delayed the patient risks developing diabetic ketoacidosis, a life threatening condition with abdominal pain, vomiting, dehydration and at the end stage reduced level of consciousness, brain oedema and death. The frequency of ketoacidosis at onset of pediatric diabetes is 15-74% worldwide [6-7]. Diabetes is, according to the American Diabetes Association (ADA), defined as a fasting venous plasma glucose of ≥7.0 mmol/L or a random plasma glucose of ≥11.1 mmol/L in a patient with symptoms typical of diabetes as polyuria, polydipsia and weight loss [8]. In the absence of typical symptoms or when random plasma glucose are equivocal, the ADA recommends an oral glucose tolerance test (OGTT), performed as described by the World Health Organisation (WHO) and the diagnosis of diabetes mellitus is established after a rise in plasma glucose to ≥11.1 mmol/L after two hours, confirmed by a high plasma glucose or a raised plasma glucose in a second OGTT another day. With values between ≥7.8 and ≤11.0 the definition is impaired glucose tolerance [8].

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both. Diagnosis of diabetes follows internationally accepted thresholds of blood glucose as mentioned above. The classification of diabetes is based on clinical symptoms and treatment requirements rather than knowledge of the etiology and pathogenesis [8]. Many diabetic patients do not easily fit into a single class, but it is less important with an exact classification as long as the child receives the correct treatment. Most children have type 1 diabetes and without insulin treatment they will die. Type 1 diabetes patients have increased risk of developing other autoimmune disorders such as celiac disease, autoimmune thyroiditis, Addison’s disease, vitiligo, autoimmune hepatitis, myasthenia gravis and pernicious anemia [8].

With modern insulin treatment the patients can have near to normal plasma glucose but it is very hard to be able to control the plasma glucose at the same level as people without the disease and increased plasma glucose levels do cause severe complications later in life. With increased plasma glucose levels for a long period the normal atherosclerosis that affects everyone accelerates and diabetic patients risk developing complications such as cardiac ischemia, retinopathy with potential loss of vision, nephropathy with renal failure and neuropathy with foot ulcers and risk of amputation. The better the metabolic control, the better the
chance for the patient to live a normal life without complications, but the lower the plasma glucose, the higher the risk for serious hypoglycaemic episodes that could lead to insulin coma or death.

Incidence and prevalence

The incidence of type 1 diabetes varies widely in the world and is rising. In children ≤ 14 years old the incidence varies from 0.1-0.6/100 000 per year in Venezuela and China to 64/100 000 per year in Finland [7, 9-10]. The rising incidence is obvious. In Finland the incidence has increased from 12/100 000 per year in 1953 to 31/100 000 per year in 1980 to 64/100 000 per year in 2005. The increase in incidence is most obvious in the younger age groups, with an increase at 4.7% more affected every year for those < 5 years old [9]. The average age at onset has decreased but the overall increase in incidence of type 1 diabetes might be due to differences in age at onset rather than to differences in the cumulative lifetime risk [9, 11]. In Sweden the incidence among children 0-14 years was 25/100 000 per year in one study between 1978-1992 and 29/100 000 per year between 1983-2000 according to another study. The average increase per year was 2.2%. The incidence increased significantly in all age groups but most in the youngest group, 3.0% in 0-4 years old [12]. The incidence in Sweden today is 44/100 000 per year [13].

The incidence varies with race, gender and age, with the highest incidence in Europe. Migrating population has the same incidence rates as other inhabitants in their new countries within a short time [7]. Most autoimmune diseases are more common in adults than in children and among women than among men, but among adult type 1 diabetes patients there are slightly more men than women. There are no large differences in incidence between boys and girls until puberty, but in high incidence countries studies have shown a minor male excess after puberty and in populations of European origin aged over 15 years the male/female ratio is 1.3-2.15 [9, 14]. However, in populations with African or Asian origin with a low incidence there is a female excess [14].

The average annual increase of type 1 diabetes in children all over the world has been 2.4-4.0% [9-10, 15]. In 1990-1994 this increase was 2.4% and during 1995-1999 it was slightly higher at 3.4%. The increase was not seen in Central America and the West Indies where the trend was a decrease of 3.6% [10, 12]. Only among the European populations did the trend in incidence diminish with age. In most
Asian populations the incidence was very low. A marked exception was Kuwait, with a high incidence of 22/100 000/year [10]. In high-incidence countries such as Finland and Sweden the lifetime prevalence is 1% [16]. During the last years there has been no increase in type 1 diabetes incidence in children and adolescents in Sweden according to the Swedish childhood diabetes register (SWEDIABKIDS) [13].

Pathogenesis

The pathogenesis of type 1 diabetes is related to autoimmunity but the detailed mechanisms are not clear. The immune system has a challenging task in recognizing and eliminating foreign antigens while at the same time tolerating the body’s own tissues (self antigen). Self-tolerance should be established during fetal and neonatal life and depends upon mechanisms controlling autoreactive T- and B-lymphocytes. The bone marrow generates immature T-cells with different T-cell receptors. On the basis of their affinity for self-antigenic epitopes and the major histocompatibility complex (MHC), also called HLA, these T-cells receive a positive signal and mature or a negative signal and get blocked in the thymus and die. The T-cells that are negatively selected in the thymus generally represent autoreactive elements and the death of these cell clones provides immunologic tolerance against self-structures [17]. This selection can fail and autoreactive T-cell clones can survive and it is thought that factors in the environment later can activate these clones. The activated T-cells attack target cells carrying self-antigens and destroy the target cells. This is called autoimmunity [17]. The reasons behind the failure of self tolerance are incompletely understood. The autoimmune destruction of insulin-producing pancreatic islet β cells leads to insulin deficiency [18].

Antibodies that are reacting against normal structures in the body are called autoantibodies. It is still unknown what comes first, if it is the autoantibodies that cause the destruction of the β cells or if the appearance of autoantibodies is only a result of and secondary to an initial insult or many insults to the pancreatic β cells. The autoantibodies might only be indicators of β cell damage, and might not play any active role in the tissue destruction [7, 16, 19]. When the β cells are destroyed, previously hidden self-antigens are exposed and activate B-lymphocytes, which amplify the initial immune response by the production of autoantibodies. The B-lymphocytes present autoantigens to T-cells and can form autoantibodies to pancreatic β cell antigens [18, 20]. In 1974 the first type 1 diabetes autoantibody,
ICA, was found [5, 21]. The researchers incubated sera from type 1 diabetes patients with multiendocrine disease with fresh frozen human pancreas and used immunofluorescence to identify the antibodies, but the antigen was not known. In 1982 Baekkeskov et al found the first antigen and in 1990 this autoantigen, which contributes to ICA reaction was found to have glutamic acid decarboxylase (GAD) activity [22-23]. In 1992, Karlsen et al cloned human islet GAD and found it to be a novel GAD isoform (GAD65) [24]. The islet antigen-2 (IA-2) is also recognized by ICA and was detected in 1995 [25-26]. Insulin was identified as an antigen in type 1 diabetes patients at diagnosis in 1983. It was already then known that insulin autoantibodies developed in patients treated with insulin. The insulin autoantibody (IAA) cannot be detected with the ICA test [27]. Finally, in 2007 the Zinc transporter 8 genetic polymorphism was found to be strongly associated with type 2 diabetes, and ZnT8A were found in type 1 diabetes patients the same year.

There is much evidence for an autoimmune process in type 1 diabetes. First, the insulitis – CD8 and CD4 T-cells as well as B-lymphocytes and macrophages, with a predominance of CD8 T-cells are present in the islets and circulating autoreactive T-cells can be detected at clinical presentation of type 1 diabetes [28-29]. However, in one study, pancreatic biopsy of prediabetic patients and patients with recent onset type 1 diabetes showed various degrees of reduction of β cell volume in all patients, but insulitis was only identified in about half of the patients [28]. Furthermore, disease progression may be delayed by immunosuppressive drugs directed specifically against T-cells or antigen-presenting cells [29]. Additionally, the appearance of islet autoantibodies to multiple islet autoantigens in most type 1 diabetes patients at diagnosis strongly supports the notion of an autoimmune process. Finally, the presence of susceptibility HLA genes and the increased propensity in type 1 diabetes patients to develop multiple organ-specific autoimmune diseases strengthen this theory [29-30].

One patient with severe B-lymphocyte deficiency has developed type 1 diabetes. This underscores that neither autoantibodies nor B-lymphocytes are essential in the pathogenesis of type 1 diabetes. The presence of autoantibodies is a prognostic marker, but the roles of the autoantibodies in the disease process are not fully understood [20].

Individuals with a family history of type 1 diabetes have an increased risk of developing type 1 diabetes compared to those without relatives with the disease. However, only about 15% of patients with type 1 diabetes have a close relative with the disease [28, 31-32]. The average risk in the population of developing type 1 diabetes is 0.4% in the USA and about 1% in Sweden [16]. There are higher concordance rates between monozygotic than dizygotic twins which suggests a significant genetic contribution. The concordance rate between dizygotic twins is
4-13% compared with 21-53% up to 70% for monozygotic twins. The largest risk for the second monozygotic twin to develop type 1 diabetes is during the first three years following the index twin’s diagnosis. Male co-twins have a higher rate of concordance than female pairs [11, 33-35]. Dizygotic twins have a higher rate of concordance for type 1 diabetes than siblings to type 1 diabetes patients, although they share the same amount of genes. Environmental exposure that initiates the autoimmune process might occur early in life when twins share a more common environment than non-twin siblings. The siblings’ risk is approximately 6-8% [11, 34, 36-38]. The fact that the concordance rate among monozygotic twins is not 100% strongly supports that environmental factors have a significant role in the pathogenesis of type 1 diabetes. Fathers are two to three times more likely to transmit the disease to their offspring than mothers. Early onset of diabetes in the parent appears to increase the risk of diabetes in the offspring [11, 14]. If the mother has type 1 diabetes the risk for the offspring is 3%, while, if the father is affected, there is a 5% risk for the child. With two affected first-degree relatives the risk is 20% [33].

The most important genes both for susceptibility towards and protection from type 1 diabetes are located within the HLA region, encoding hundreds of genes but the most important are the HLA-DR and HLA-DQ [3-4]. However, less than 10% of individuals with HLA-conferred diabetes susceptibility progress to clinical disease. The HLA locus is thought to confer approximately 50% of the genetic susceptibility for type 1 diabetes. There is strong linkage disequilibrium between the neighbouring genes in the HLA complex. HLA-DR modifies the risk conferred by HLA-DQ [7, 11, 28-29, 38-44].

In addition to HLA, more than 40 non-HLA genetic factors have been proposed to be associated with type 1 diabetes and are thought to be important regulators of immune response but to have only modest individual effects on the risk [44]. The role of these genetic factors to affect the risk for type 1 diabetes remains to be clarified. They were initially named insulin-dependent diabetes mellitus genes, with a number - IDDM2, IDDM3 etc, and are located on different chromosomes [7, 28, 43-44]. However, as the list grew larger this nomenclature was abandoned and replaced by the name of the gene or the location in the genome in case a genetic factor was not immediately identified. About 15% of the risk is explained by two of these genes, the insulin gene (INS or insulin-VNTR or IDDM2) located on chromosome 11p15 and the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4 or IDDM12) located on chromosome 2q31-33. CTLA-4 modulates immune responsiveness in its role in T-cell development and antigen recognition [7, 44]. The INS gene confers additional risk of developing type 1 diabetes above that of HLA, regardless of HLA genotype [45]. In the INS gene a variable number of tandem repeats (VNTR) from about 26 to over 200 are found and these affect
the risk of developing type 1 diabetes. The short class I VNTR alleles and the I/I INS VNTR genotype are associated with an increased risk of type 1 diabetes, whereas there is a decreased risk of the disease with the long class III alleles and I/III and III/III genotypes [38, 45]. This gene is thought to influence the autoimmune target specificity [45]. The I/I, I/III and III/III genotypes were found in 75%, 23.2% and 1.8% of patients with type 1 diabetes compared to 56%, 37.2% and 6.8% of control subjects [45].

The genetic susceptibility in family members depends on the degree of genetic identity with the proband and the number of shared alleles. There is also an interaction between the different type 1 diabetes susceptibility loci. The risk of developing type 1 diabetes in a child who has a sibling with the disease can be stratified from 0.3% up to 30% depending on the child’s HLA class II genotype. Type 1 diabetes risk in children without a family history of type 1 diabetes can be stratified from ~0.01% to more than 5% depending on the HLA genotype [33, 41, 46]. The risk can also be estimated empirically on the basis of the frequency of the HLA genotype. The most important change in type 1 diabetes risk status of a child occurs when islet autoantibodies develop [33].

Continuing destruction of β cells leads to progressive loss of insulin-secretory capacity with loss of pulsatile insulin secretion and first phase insulin response (FPIR) in intravenous glucose tolerance test (IvGTT) and increased 2h glucose in OGTT [47]. Rising HbA1c levels and increased insulin resistance have also been observed prior to type 1 diabetes onset [48-49]. However, fasting plasma glucose does not have a predictive value [47]. Type 1 diabetes is usually diagnosed when insulin secretion falls below a critical amount, with about 20% of the β cells left, and progresses to a state of absolute insulin deficiency in most but not all patients [29].

**HLA**

The HLA class II genes are involved in antigen presentation, as they affect the peptide binding groove and define which peptide will be bound and presented to T-cells. This variability is probably the mechanism behind the positive and negative disease associations of specific HLA class II molecules and the HLA-DQ alleles determine autoantibody expression [16, 39, 50]. In the beginning HLA were named after their DR haplotype but today most information is given about the HLA-DQ alleles (Figure 1). In most cases it is only necessary to define the
DQB1 allele, as the other allele can be deduced from the strong linkage disequilibrium. Disease risk can be assigned to a given haplotype or genotype [39-40].

![Figure 1. HLA class II region on Chromosome 6](image)

The positive associations between HLA and type 1 diabetes are not absolute and decrease in strength with increasing age at onset. The negative association does not eliminate the possibility of developing type 1 diabetes [16]. It is controversial whether HLA influences the development of positive autoantibodies, the subsequent progression to type 1 diabetes or both stages [50]. The absolute disease risk associated with different HLA Class II genotypes ranges from 5-8% to less than 0.01% [40]. DQ molecules are formed by two polymorphic genes (α and β) and each amino acid variant of α and β is given a four digit number preceded by the gene designation (e.g. DQB1*0302) [51].
The HLA-DQB1*0302 allele is in linkage disequilibrium with DQA1*0301 and DRB1*04. The major effect contributed by the HLA-DR locus is seen in the variability of diabetes risk conferred by HLA-DR4 positive HLA-DQB1*0302 haplotypes depending on the specific DR4 subtype. The HLA-DRB1*0403 and *0406 alleles in the haplotype are protective and *0401, *0402, *0404, and *0405 are associated with variable degree of risk [32, 39-40]. DQB1*0602 has a strong dominant protective effect and further association is not meaningful to analyze. This haplotype is found in 20% of the US population compared with less than 1% of children who develop type 1 diabetes [16, 38]. HLA-DQB1*0301 may be associated with DQA1*0301 (the DR4 haplotype) or DQA1*0501 (the DR5 and DR6 haplotypes), both without diabetes risk. It is important to analyze the DQA1 allele associated with DQB1*02 as the association with DQA1*0201 (the DR7 haplotype) gives a protective or neutral haplotype but the association with DQA1*0501 (the DR3 haplotype) or DQA1*0301 gives a diabetes risk haplotype [32, 39-40, 50]. There is a protective effect of DQA1*0201-DQB1*0303 (in linkage disequilibrium with DR7) and DQA1*0101-DQB1*0503 (DR14) for autoantibody expression but no significant difference in diabetes risk [50].

The HLA-DQA1*0501-DQB1*0201 (DQ2) and HLA-DQA1*0301-DQB1*0302 (DQ8) haplotypes are high risk haplotypes and the HLA DQ 2/8 genotype encodes the highest risk for type 1 diabetes. This genotype is only found in about 2.3% of the white population compared to 24-30% of type 1 diabetes patients [32, 38, 44, 52]. Children with this genotype have a risk of approximately 1 in 20 for developing type 1 diabetes before the age of 15 years, and if the child has a sibling with type 1 diabetes and this haplotype the risk increases to 55% [44]. According to previous studies of type 1 diabetes patients at diagnosis 32% of the patients carried the DQB1*0302 allele. The *0302/X (X is not *02) genotype was most common, found in 48%, and the *02/Y (Y is not *0302) genotype was found in 17% [32, 52]. About 90-95% of the Caucasian population with type 1 diabetes have DQ2 or DQ8 haplotypes, compared with 40-50% in the control subjects [16, 52].

Environment

Dizygotic twins and siblings share the same amount of genes, but the concordance rate of type 1 diabetes for dizygotic twins is double that of siblings. Dizygotic twins are thought to share the environment more than siblings, supporting that environmental factors affect the risk of type 1 diabetes. Many environmental risk
factors have been identified but most are controversial. About 20% of children born with congenital rubella develop type 1 diabetes. Other viral infections such as enteroviruses, rotavirus, parvovirus and cytomegalovirus are found more often in type 1 diabetes patients. The question is to what extent they affect the disease process, initiation and/or progression [11, 16, 28].

Type 1 diabetes incidence increases after epidemics due to enteroviruses, especially Coxsackie virus, and entero viral RNA can be detected in the blood of >50% of type 1 diabetes patients at diagnosis. Enteroviruses can infect isolated human pancreatic β cells, with functional damage or cell death as a result [53]. There are more than 100 defined human enterovirus serotypes and most enterovirus infections are asymptomatic. However, virus found at diagnosis may have infected the host a long time after the disease process started and may not explain the disease process. There is also a possibility that etiological infections have been cleared at the time of diagnosis and that we miss the real cause of the disease. If an infection would be the etiology to the autoimmune process, it should occur just before islet autoimmunity is found. Differences in serum enterovirus RNA in the period before islet autoimmunity compared to matched controls have not been reported. However, the rate of progression to type 1 diabetes was significantly higher after detection of entero virus RNA in serum in children with islet autoantibodies, but in the same study none of the samples from the day of type 1 diabetes diagnosis were positive for enterovirus RNA [54].

Other viruses may protect against the development of type 1 diabetes and different serotypes of the same virus may protect or be a possible trigger of the disease. Coxsackievirus-B4 is able to infect human thymus in vitro and is thought to disturb the maturation/differentiation processes, sometimes with persistence of the virus in the host [42].

Nutritional factors are also thought to increase the risk of type 1 diabetes. Early or late (<3 or >7 months of age) introduction of gluten as well as early exposure to cow’s milk proteins, often associated with the lack of or short time of breast-feeding, high nitrate intake and low vitamin D or omega-3 fatty acid intake have been associated with increased risk of type 1 diabetes, but not obvious in every study [55-56]. Early introduction of fruits, berries and root vegetables are also believed to increase the type 1 diabetes risk [57].

As childhood obesity is rising in parallel with type 1 diabetes incidence, rapid growth and weight gain is also thought to increase type 1 diabetes risk [58]. According to the ‘accelerator hypothesis’ increased weight gain causes increased insulin resistance. The increased blood glucose accelerates β cell apoptosis
directly and through the autoimmune attack in genetically predisposed individuals [59].

Perinatal factors such as greater maternal and paternal age at delivery also affect the risk of developing type 1 diabetes. A mother giving birth after the age of 40 is up to three times more likely to have a child who develops diabetes than a mother in her twenties [14]. Blood-type incompatibility between the mother and child, preeclampsia, maternal infections during gestation, large birth size, lower gestational age, earlier birth order and complicated birth are all considered as type 1 diabetes risk factors [60-61]. Psychological stress has also been suggested as a risk factor, though hard to measure in retrospective studies [62].

According to the ‘Hygiene hypothesis’, the decreased frequency of childhood infections, caused by improved hygiene and living conditions, leads to a modulation of the developing immune system and an increased risk for autoimmune and allergic diseases [63]. Autoimmune diseases are more common in smaller families and in children not in day care. These children are less exposed to infections or other immune challenges early in life that stimulate the immune system and in that way has a protective influence [7, 64].

Furthermore, type 1 diabetes develops more frequently in winter months than in summer months and is more common among children born during spring than fall [65-66]. There is a large geographic variation in type 1 diabetes incidence, with the highest incidence in countries that are further from the equator where the winters are colder and some viral infections are more common [67].

Low serum zinc levels are common in diabetes and zinc supplementation can inhibit the development of experimental diabetes in mice [30]. Both high and low serum zinc levels have been found in type 1 diabetes patients [68]. According to a former study plasma zinc at type 1 diabetes diagnosis and up to two years after did not differ significantly with the zinc levels in controls and did not correlate with the quantitative changes in β cell function [69].

Islet Autoantibodies

The appearance of autoantibodies is the first detectable sign of β cell autoimmunity. There are five major autoantigens in type 1 diabetes against which autoantibodies are produced: islet cell proteins, glutamic acid decarboxylase
(GAD), insulinoma associated protein 2 (IA-2), insulin (IA) and the zinc transporter 8 (ZnT8). Islet autoantibodies can be measured for all these antigens (ICA, GADA, IA-2A, IAA, ZnT8RA, ZnT8WA and ZnT8QA). These autoantibodies can be transferred from the mother during pregnancy and disappear slowly (usually at 6-9 months of age) [70-71]. Islet autoantibodies can develop at any age and have turned out to be present as early as the first few months of life, but most commonly develop between 9 months and 3 years of age [28]. The first islet autoantibody to appear in infants is usually IAA [70]. About half of the young children under five years of age have IAA as their first islet autoantibody, and about 1/4 show ICA first. GADA is more common than IA-2A, which is the least common first islet autoantibody in young children [72].

Islet autoantibodies usually appear sequentially and rarely simultaneously and they can occur late in life, although the appearance of a second autoantibody most often occurs within a relatively short time. If the patient does not develop other autoantibodies within a year after the appearance of the first autoantibody, this rarely occurs later [29, 73-74]. Progression to diabetes from the development of islet autoantibodies differs between individuals, from months to more than 20 years and according to one study with a median of 8.6 years [51, 73, 75-77]. It has been suggested that the time from seroconversion to clinical onset has been shortened [78]. The age when the first autoantibody appears is associated with the rate of progression to diabetes; the younger the child is at first autoantibody seroconversion, the younger the child is at clinical onset [73, 78].

A single autoantibody is found in about 4% in the general population and is mostly not accompanied by the development of type 1 diabetes but might instead reflect harmless transient non progressive or even regressive β cell autoimmunity in about half of such individuals. Multiple autoantibodies are usually persistent and represent, in most cases, destructive progressive β cell autoimmunity [29, 51, 72-73]. Islet autoantibodies can both appear and disappear and inverse seroconversion is common, more so for IA-2A than GADA [73]. Some indications exist that the loss of IA-2A might increase the risk of progression to type 1 diabetes [79]. Both the positive and the negative seroconversions reflect the dynamic process of β cell autoimmunity [73]. Those individuals who lose their autoantibodies can progress to type 1 diabetes disease. Half or more of siblings to type 1 diabetes patients with autoantibodies have been shown to regress with no signs of prediabetes at the end of follow-up [80]. Positivity for multiple autoantibodies is rare in the general population. Less than one in 300 in the general population express two or more autoantibodies [51]. A single-point negative autoantibody test was associated with a low risk of developing type 1 diabetes in the general population [51, 76].
The risk of type 1 diabetes significantly and progressively increases with an increasing number of autoantibodies, regardless of HLA genotype [50]. At type 1 diabetes diagnosis multiple autoantibodies, three or more were observed in 73-77% of the patients and those who were positive for multiple autoantibodies tended to be younger [19, 52]. Individuals expressing two or more of the autoantibodies almost always progress to type 1 diabetes given long enough follow-up, both in family studies and in studies of the general population. About 50-80% and according to one study 100% of children positive for two or more islet autoantibodies developed type 1 diabetes during an observation period of 5-15 years. With an increasing number of autoantibodies the time to type 1 diabetes diagnosis tended to be shorter [29, 51, 75-77, 81]. A one-time screening for GADA and IA-2A could identify ~60% of the individuals who developed type 1 diabetes over the subsequent 27 years [73].

The level of the different islet autoantibodies has been shown to influence the risk of progression to type 1 diabetes in some studies, but not in others. High ICA-levels, i.e. greater than 20 Juvenile Diabetes Foundation Units (JDF-U), were associated with a higher risk of progression to disease. This has also been shown for higher levels of IAA and IA-2A but not for GADA [31, 73, 79, 81-82]. A high ICA level most often represents multiple autoantibodies which may explain the increased risk as multiple autoantibodies are associated with increased risk of developing type 1 diabetes. The presence of multiple autoantibodies is far more strongly associated with the risk of type 1 diabetes than is a high titer of autoantibody to any single antigen [16, 76, 81, 83]. It is possible that the association between multiple autoantibodies and high titers of the autoantibodies is a function of the stage of progression to type 1 diabetes [81].

According to one report analyzing ICA, GADA, IA-2A and IAA, 36% of type 1 diabetes patients were positive for all 4 autoantibodies at clinical onset, 37% were positive for 3 autoantibodies, 19% for 2 autoantibodies, 6% for 1 autoantibody and 2.3% were autoantibody negative. The autoantibody negative patients were older at diagnosis [19]. Previous studies have shown that some of the autoantibody negative patients had cord blood islet autoantibodies from their mothers [71]. An alternative explanation to why patients are autoantibody negative at clinical onset is that they have been positive for one or more autoantibodies earlier but seroconverted to negativity before diagnosis [73].

The combination of high-risk HLA genes with autoantibodies further increases the positive prediction and autoantibodies appear earlier in children with high-risk genes [16, 72, 84]. The highest risk genotype HLA DQ2/8 is significantly less frequent among the islet autoantibody negative children and the protective allele DQB1*0602 is significantly increased among autoantibody negative children and
adolescents [85]. ICA-positive relatives to type 1 diabetes patients with DQB1*0602 extremely rarely progress to disease [84]. Individuals carrying the DQ2 genotype less often test positive for multiple autoantibodies [52]. However, according to another study, in relatives with multiple autoantibodies, type 1 diabetes risk or protection was not further determined by HLA [50].

All autoantibodies are useful predictive markers for type 1 diabetes development. Young age at seroconversion, high ICA level, multipositivity, GADA positivity, IA-2A positivity, IA-2A level and persistent positivity for IAA were significantly associated with increased risk for type 1 diabetes and the most specific predictor was IA-2A [72-73, 86]. The diabetes risk was higher in patients with ZnT8A than in those without ZnT8A even when they only displayed one of the other islet autoantibodies [87].

The prevalence of islet autoantibodies in young siblings to type 1 diabetes patients with high risk HLA is high; 35% had at least one autoantibody and 6-29% had two or more autoantibodies [72, 77]. According to another report, in offsprings to parents with type 1 diabetes, 7.5% had one autoantibody and 3.5% more than one autoantibody by two years of age regardless of HLA-defined genetic risk [70].

ICA

Islet cell antibodies (ICA) were described already in 1974 [5, 21]. ICA can be detected by the reaction of patient serum with sections of human pancreas and then by using immunofluorescent staining for these autoantibodies. The ICA assay is hard to standardize and reproduce as it is labour intensive, operator dependent and requires human pancreas that needs to be extensively tested before it can be used for analytical purposes. International standardization programs have been used to improve the quality of the analysis [90].

When GADA was discovered [23] it was first thought to be the main ICA antigen but further studies revealed that other antigens must be present and when IA-2A was discovered [25], researchers thought that IA-2A together with GADA could replace the more complicated ICA analysis. Further studies proved that there would be yet other antigens as the combination of ICA, GADA and IA-2A identified more patients, 94%, compared to 87% with ICA alone and 89% with the combination of GADA and IA-2A [88]. According to another report the ICA
reactivity could not be blocked completely by GADA, IA-2A and IAA, also suggesting that other antigens can be involved in the ICA reaction [89].

At clinical onset of type 1 diabetes in children, adolescents and young adults, ICA can be detected in 73-84% [52, 91-93], compared to 1.4-4% in children in the general population [77, 92-95], and 8% among siblings to type 1 diabetes patients [77].

ICA is the most sensitive single marker, while as many as 81% of the patients developing type 1 diabetes display ICA before clinical onset [77]. The specificity though is not as high, and 5% of siblings who did not develop type 1 diabetes were ICA positive. ICA in the absence of other islet autoantibodies did not predict future type 1 diabetes, according to another publication, and may represent a phase of harmless β cell autoimmunity but may later turn into destructive β cell autoimmunity [72, 77].

As mentioned above, high ICA titers are associated with an increased risk of type 1 diabetes [77]. Children with high ICA titers are more often positive for GADA, IA-2A and IAA and more often display multiple autoantibodies than those with low ICA titers [77, 83-84, 86]. Children with ICA, GADA, IA-2A and IAA had higher ICA titers than those with three of these autoantibodies, who had higher than those with two, who had higher than those with one [84]. An isolated low level of ICA positivity was not associated with type 1 diabetes risk in one report [86]. The frequency of ICA is similar in boys and girls, but the boys tend to be younger at seroconversion. ICA is inversely related to age as positive patients are more often under 10 years of age (88% vs. 77%) [52, 77]. Combined screening for IA-2A and GADA could identify 70% of all ICA-positive siblings [77].

Children with a high-risk genotype seroconverted to ICA positivity at a younger age and the ICA titer was higher in those with a high-risk genotype [84]. ICA was significantly associated with the DQA1*0301 allele and the DQ8 haplotype and negatively associated with DQ2 in the absence of DQ8 [52, 92, 96].

The persistent association between ICA positivity and diabetes risk, after adjustment for positive tests for all other autoantibodies including ZnT8A, implies the existence of other as yet unidentified autoantibodies to specific antigens [87].
Glutamic acid decarboxylase (GAD) is the enzyme that synthesizes γ-amino butyric acid (GABA) from glutamic acid. In 1990 autoantibodies to this enzyme, GADA, were first described by Baekkeskov [23]. GAD exists as two isoforms, GAD65 and GAD67, according to their molecular weights, and these are encoded by different genes [97]. Both isoforms are expressed in neurons and pancreatic islet cells and are approximately 65% identical. The GAD65 isoform was found in 1991 and the gene is found on chromosome 10p11 [24]. GAD65 predominates in pancreatic β cells, but the function in the β cells is not fully clarified, and it is also the isoform used in autoantibody analysis for type 1 diabetes. High titer GAD65 autoantibodies in diabetes sera cross-react with GAD67 [16]. Autoantibody reactivity to GAD67 is more common in Stiff Person Syndrome because of exceedingly high titers [97].

At clinical onset of type 1 diabetes in children and adolescents GADA can be detected in 68-73% [52, 92, 98], compared to 2-4% in children from the general population [92, 95] and 7% of siblings to type 1 diabetes patients [77]. According to a study of siblings to children with type 1 diabetes, 69% of the siblings who progressed to type 1 diabetes displayed GADA, whereas 4% of the nonprogressors displayed GADA. The levels of GADA did not differ between progressors and nonprogressors [77].

GADA were more frequent in children older than 10 years (79% vs. 69%) [19, 52]. GADA levels were associated with female sex, an effect seen in children of all ages [19, 52, 92], although not reproducible in all studies [73].

Looking at autoantibodies after clinical onset of type 1 diabetes, according to one study, it was less common for children to be in clinical remission at 18 months after diagnosis if they were GADA-positive [19]. GADA positivity tends to be stable after clinical onset of type 1 diabetes [16]. GADA is the autoantibody that predominates in adults with autoimmune diabetes [16].

GADA is significantly associated with both the DQA1*0501 and DQB1*0201 alleles and with the DQ2 haplotype, but not with the DQA1*0301 and DQB1*0302 alleles or the DQ8 haplotype unless present in combination with a DQ2 allele. Hence there is a positive association with the high risk DQ2/8 genotype. [45, 52, 84, 92, 99]. Children carrying DQB1*0302/X genotypes have lower GADA levels at diagnosis [52].
Inverse seroconversion for GADA is common and even more common among females in the general population. One third of the subjects initially positive for GADA tested negative 6 years later. The GADA titers did not differ between the progressors and nonprogressors [73].

After diagnosis the GADA prevalence decreased progressively, but there was no significant decline in GADA titer during follow-up. On the contrary, GADA positive individuals frequently showed increases in titer over time [100-101].

*IA-2A*

Insulinoma associated protein 2 (IA-2) is an unusual member, which lacks enzymatic activity, of the transmembrane protein tyrosine phosphatase (PTP) family and is located on chromosome 2q35 [16]. IA-2A was first described in 1994 by Lan et al [102]. The protein is expressed in neuroendocrine tissues and in the pancreas in both α and β cells. It is localized to the secretory vesicles in both endocrine and neuronal cells. The function is not known [16]. Another closely related protein, also an antigen in type 1 diabetes, is phogrin or IA-2β, located on chromosome 7q36, which is also expressed in neuroendocrine tissues. IA-2 is the protein of choice for most immunoassays as most sera that recognize IA-2β also recognize IA-2, but not all sera that recognize IA-2 recognize IA-2β [16].

At clinical onset of type 1 diabetes in children and adolescents IA-2A can be detected in 72-86% [52, 98], compared to 0.8% in a young adult general population [91] and 5% among siblings to type 1 diabetes patients [77].

A previous study could not find any difference in mean age or sex distribution between IA-2A positive and negative patients [19]. In another report boys had higher titers than girls of IA-2A [73]. IA-2A might be related to rapid progression to type 1 diabetes [72]. According to a study of siblings to children with type 1 diabetes 69% of the siblings who progressed to type 1 diabetes displayed IA-2A, whereas only 2.5% of the nonprogressors displayed IA-2A. The progressors had higher levels of IA-2A and were significantly younger than the nonprogressors [77]. IA-2A had the highest positive predictive value (55%) for the development of type 1 diabetes [72, 103]. Children with IA-2A had lower serum C-peptide
concentrations than IA-2A negative patients two years after clinical onset and therefore needed more exogenous insulin [19].

Children carrying DQ8 genotypes had higher IA-2A levels, whereas children carrying the DQ2 genotype had a decreased frequency and levels of IA-2A [52, 96, 99]. IA-2A had the highest titers in those with moderate and not high risk HLA [84].

According to one study, inverse seroconversion for IA-2A (57%) was even more common than for GADA but without sex differences in the general population. IA-2A tended to decrease with the duration of type 1 diabetes disease [16].

The IA-2A titer declined significantly after diagnosis in type 1 diabetes patients [100-101].

**IAA**

In 1983 Palmer et al found antibodies against endogenous insulin in type 1 diabetes patients before the start of insulin treatment [27]. They were already known to develop after insulin therapy [104]. The insulin gene is located on chromosome 11p15 [16]. Current assays cannot separate IAA that have developed before exogenous insulin treatment from IAA that develop after the start of treatment, so IAA analysis is not useful for confirming the classification of diabetes after insulin therapy has begun [16]. The development of IAA after the start of insulin treatment is fast; after one to three weeks four of five patients tested IAA positive and had significantly higher autoantibody levels than IAA positive patients had within one week after start of insulin treatment [105]. Insulin is an autoantigen that is specific to β cells, compared to GAD and IA-2 which can be detected in other tissues.

At clinical onset of type 1 diabetes in children and adolescents IAA can be detected in 43-56% [19, 52, 92, 98] and in 91% of the children under four years of age [106-107], compared to 1-3% in children in the general population [92-93, 95] and 4% of siblings to type 1 diabetes patients [77].

According to a study of siblings to children with type 1 diabetes, 25% of the siblings who progressed to type 1 diabetes displayed IAA, compared to 3% of the
nonprogressors. The progressors had higher levels of IAA and were significantly younger than the nonprogressors [77].

Children that were IAA positive were younger, on an average two years younger, at diagnosis compared to IAA negative patients [19, 92]. IAA was detected almost twice as often in children under the age of five years (74% vs. 41%) [52]. The prevalence of IAA at type 1 diabetes diagnosis was higher in males than females between 15 and 21 years of age [108].

Children that were IAA positive had lower C-peptide levels at follow-up for up to two years after type 1 diabetes diagnosis compared to IAA negative patients, and needed more exogenous insulin two years after clinical onset. A lower proportion of the IAA positive patients were in clinical remission one year after diagnosis [19].

IAA is associated with DQA1*0301/DQB1*0302 alleles and the DQ8 haplotype. In the absence of DQ8, IAA is negatively associated with DQ2 [29, 52, 92, 96, 99]. Hence, there is a positive association between IAA and the high risk DQ2/8 genotype.

IAA were transient more frequently than the other autoantibodies and fluctuated between positivity and negativity more often than ICA [72]. With a high titer of IAA there was a larger probability that the individual would remain IAA positive [72]. A decline of IAA at older age at clinical diagnosis was frequent [70].

ZnT8A

Since 1934 it has been known that zinc permits the formation of insulin crystals and that zinc-deficient animals have a lack of insulin in β cells [68, 109]. Zinc is an important structural component of many proteins, used as a cofactor for many enzymes and a transcription factor, which mediate key cellular processes, such as protection against oxidative stress, DNA repair, DNA replication, proliferation, differentiation and apoptosis. Zinc plays an important role in the immune system. The body has only limited zinc stores and depends on daily zinc intake. Approximately 20% of the world’s population has an inadequate zinc intake, and impaired zinc homeostasis is linked to several diseases besides diabetes mellitus. Diabetic patients have an increased urinary excretion of zinc compared to healthy
controls. Plasma zinc is often low in type 2 diabetes patients but high in type 1 diabetes patients, with higher concentrations at the beginning of the disease, when the destruction of β cells takes place [68, 109]. Zinc supplementation can improve glycaemia in both type 1 and type 2 diabetes and inhibit the development of type 1 diabetes in mice [30, 68, 109-112]. The highest levels of zinc in the body are found in the pancreatic β cells where free zinc is concentrated in the insulin secretory granules and is essential for the storage, secretion and the action of insulin [113]. Zinc homeostasis is regulated by zinc transporters - ZnT (SLC30A gene family) and ZIP (SLC39A gene family). The ZnT decrease intracellular zinc levels by transporting zinc ions out of the cytoplasm into extracellular spaces or into secretory granules, while the ZIP increase cytoplasmic zinc by transporting zinc ions from extracellular spaces into the cytoplasm. In humans 10 members of the ZnT family (ZnT1 to ZnT10) and 14 members of the ZIP family (ZIP1 to ZIP14) are currently known. High glucose has no effect on the expression of ZnT8, but ZIP are upregulated. Cytokines (interleukin-1β) and tumor necrosis factor (TNF)-α decrease the expression of zinc transporters, particularly ZnT8 [109-111, 114].

In 2007, genome-wide association studies (GWAS) found that a single nucleotide polymorphism (SNP) in the gene SLC30A8, which is coding for zinc transporter 8 (ZnT8), was strongly associated with type 2 diabetes susceptibility [115-116]. This gene was discovered already in 2002 and is located on chromosome 8 at position q24.11 and mostly expressed in islets of Langerhans in the pancreas [30, 116]. The gene contains eight exons and encodes a 369-amino acid ZnT8 protein [98, 116]. The ZnT8 protein has six transmembrane helixes with a histidine-rich domain between the fourth and the fifth helix (Figure 2). ZnT8 is quite specific for the β cells and localized in insulin containing secretory granule membranes and is responsible for transporting zinc into the granule [30, 116-118]. Zinc is required for hexamirization and conversion of proinsulin to insulin. Proinsulin interacts with zinc ions to form a zinc$_2$ proinsulin$_6$ hexamer, later converted into zinc-insulin hexamers in secretory vesicles. Proinsulin hexamers are water-soluble in physiological zinc concentrations, whereas insulin hexamers are water-insoluble and zinc-insulin crystals are formed. More zinc ions (4:6) are present in the zinc-insulin crystals than the 2:6 ratio in hexamers. This extra zinc, which is required to displace water molecules between insulin-zinc hexamers, is delivered through the ZnT8 [110]. Zinc is co-secreted with insulin from the secretory granules during exocytosis. Zinc is released from insulin when it reaches the higher pH of blood. The rise in zinc ions inhibits glucagon release from the α cells [118]. ZnT8 is also expressed in α cells of pancreatic islands, peripheral blood lymphocytes, subcutaneous fat tissue, thyroid follicles and the cortex of the adrenal gland [111-112, 118]. The role of ZnT8 in the pathogenesis of type 1 diabetes is still unknown.
Figure 2. The structure of the ZnT8 protein with six transmembrane regions, a histidine-rich domain. Amino acid 325 in the ZnT8 is a key determinant of the autoreactivity, (adapted from Kawasaki 2012 [30]).

Zinc is an essential trace element for immune function and modulates the expression of many genes within the immune cells. Zinc is important for the generation of proinflammatory cytokines, such as interleukin-6 and TNF-α, and the development of T-cells and B-cell apoptosis as well as B-cell response [30, 68]. Zinc deficiency induces thymic atrophy, lymphopenia, and suppression of cytolytic T-cell responses, natural killer cell activity and delayed-type hypersensitivity reactions. Zinc protects pancreatic β cells from cytokine-induced destruction. The zinc content of β cells is high because of the overexpression of ZnT8 which can protect the β cells from apoptosis related to zinc depletion [30].

At clinical onset of type 1 diabetes in children and adolescents ZnT8A can be detected in 24-80%, with the lowest levels of 24% and 58% in Chinese and Japanese children [30, 98, 115, 119-122]. It is found in 19% of adults with adult onset type 1 diabetes with GADA or IA-2A and 1.4% of type 2 diabetes patients.
ZnT8A is found in <3% of control subjects and in 1.9-4.6% in healthy siblings to type 1 diabetes patients [98, 115, 121, 123-124].

A SNP, rs13266634 at position 325 of the ZnT8 encoding gene, causes an amino acid replacement from arginine (CGG) to tryptophan (TGG). Another SNP (rs16889462) at the same position codes for glutamine (CAG). The specificity of the ZnT8A response is reflected by the rs13266634 genotype [101, 124-125]. Arginine (R) is encoded by the C allele and tryptophan (W) is encoded by the T allele [121]. The CC genotype, the dominating genotype, is highly associated with ZnT8RA and the TT genotype (only found in 8-12%) is highly associated with the ZnT8WA subtype. The heterozygous carriers (CT) have intermediate values for both ZnT8RA and ZnT8WA. The ZnT8WA has been found in a few CC genotype carriers, which indicates that the specificity is not 100% [122, 125-126]. This indicates that amino acid 325 greatly affects a major antibody epitope but that the restriction is not absolute [101]. Glutamine (Q) shows no significant variation with the rs13266634 genotype [125].

ZnT8RA is the most common ZnT8A in Europeans and African-Americans. ZnT8WA is almost twice as common in Asians as in Europeans and very rare in African-Americans [101]. ZnT8RA was found in 53%, ZnT8WA in 44% and ZnT8QA in 34% of children and adults 0.6-58 years of age with newly diagnosed type 1 diabetes [125].

The ZnT8A prevalence was low in the very young children, according to one study, but increased with a peak of 80% in late adolescence and declined thereafter [122]. ZnT8RA was more common after five years of age while the ZnT8WA prevalence was independent of age at onset. The youngest patients had the lowest ZnT8RA levels, whereas there were no age differences for ZnT8WA levels [122].

About 21% of children with type 1 diabetes reacted to all three ZnT8A at diagnosis. ZnT8QA was rarely found without the other ZnT8A [120]. Although ZnT8A appeared later than the other autoantibodies in many children during the preclinical phase they seemed to identify individuals with a more rapid progression to clinical onset, as was previously described for IA-2A. ZnT8A might also be present many years prior to clinical onset of type 1 diabetes [98, 115, 117]. ZnT8A have been found in children as early as at two years of age. GADA and IAA tended to appear earlier than IA-2A and ZnT8A, but there was no strict order of appearance of autoantibodies. ZnT8A tended to persist to diagnosis. The combined measurement of ZnT8A, GADA, IA-2A and IAA raised autoimmunity detection rates to 98% at disease onset [115, 125].
ZnT8RA and ZnT8WA levels declined significantly during the first years after clinical onset, similar to C-peptide [100-101]. About 40% of the ZnT8A positive patients became negative during a follow-up of five years after diagnosis [101]. Only 7% were ZnT8A positive after 25 years compared with 20% for IA-2A and 26% for GADA. No patients have been found so far to develop ZnT8A after clinical onset [100]. In some patients the C-peptide levels were sustained despite the ZnT8A decline. Each individual maintained the same pattern of reactivity to the ZnT8A suggesting that major epitope switching or spreading do not occur [100].

It is not clear why autoantibodies persist despite the loss of β cells as the antigen source after disease onset. The half-lives of the ZnT8A, GADA and IA-2A after disease onset exceed the half-life of human immunoglobulin molecules in the circulation, suggesting that the decline in autoantibody titer reflects waning of autoreactivity rather than cessation of autoantibody production [100]. Another theory is that the ZnT8A is a potential marker for islet destruction. As IA-2 and GAD65 are expressed in tissues other than pancreatic β cells while ZnT8 is expressed mainly in β cells and α cells, the declining ZnT8A titers during the continuous autoimmune destruction of pancreatic β cells after diagnosis, with a simultaneous fall in C-peptide, may support this theory. There seems to be a reactivation of GADA autoimmunity [100].

Autoantibody responses in any individual appear polyclonal. The structural variation in the antibody repertoire that occurs between genetically identical individuals is pronounced. Despite this, epitope restriction may occur in autoimmunity. The humoral type 1 diabetes autoimmunity to ZnT8 is thought rather to be directed against self than nonself epitope determinants. Amino acid 325 in the ZnT8 is a key determinant of two of the three major conformational epitopes in the protein. The different responses to the three different ZnT8A in genetically similar individuals argue against the molecular mimicry hypothesis. In this hypothesis, autoimmunity is thought to be triggered by an initial immune response to an infectious agent that triggers reactivity to self because of sequence homology between the pathological agent and a self protein. The mimicry model would more likely favor one epitope over another. However, amino acid 325 may be represented by three different amino acids. Each amino acid (R, W or Q) may yield a specific autoantibody response. It is therefore speculated that ZnT8 autoreactivity rather arises because of a defect in induction of self-tolerance than because of molecular mimicry [125].
VAMP2 and NPY

Two additional minor autoantigens in type 1 diabetes patients are the vesicle-associated membrane protein 2 (VAMP2) and neuropeptide Y (NPY), found in 21% and 9%, respectively [127]. The search for additional autoantigens continues, but is out of the scope of this thesis.

HbA1c

Red blood cells contain hemoglobin (Hb) of three variants. HbA dominates but there is also HbA2 and HbF. There are also subcategories of HbA called glycated hemoglobins, A1a, A1b and A1c, and HbA1c is the major fraction. Glycation is the nonenzymatic addition of sugar residues to amino groups of proteins. HbA1c reflects the mean glucose concentration during the life span of the red blood cells and is an indicator of the metabolic control during the preceding approximately 6-8 weeks. An increase in HbA1c, even within the normal range, can predict an increased risk of progression to type 1 diabetes, independent of random glucose and number of islet autoantibodies. Even in the absence of chronic hyperglycemia, postprandial or other temporary hyperglycemic episodes may influence HbA1c within the normal range [48].

C-peptide

The information on normal stimulated C-peptide values in young children is limited as is information about fasting C-peptide, but the youngest children most often have lower fasting C-peptide than older children and adults, and C-peptide levels tend to be higher in females, which might be explained by insulin resistance [128]. Studies have shown little change in fasting C-peptide, peak C-peptide and area under the curve (AUC) C-peptide in OGTT during 30 months before type 1 diabetes diagnosis and at diagnosis, but a decreased early C-peptide response and
an increased late C-peptide response at least two years before type 1 diabetes diagnosis [129-130].

C-peptide is often detectable two years after type 1 diabetes diagnosis and there is a biphasic decline in C-peptide after clinical onset, best measured as a peak value or AUC and not as a fasting C-peptide [131-132]. It is thought that a residual insulin and C-peptide secretion reduces the risk of future micro-vascular complications and serious hypoglycemia. C-peptide is a hormone that might have important effects in itself, not just reflecting residual insulin secretion [133].

Patients with no detectable autoantibodies at diagnosis tend to have higher serum C-peptide concentrations after clinical onset and need less exogenous insulin than autoantibody positive patients. IA-2A positive patients often have reduced serum C-peptide concentrations at follow-up and a higher daily insulin dose compared to IA-2A negative patients [19]. According to a Danish study, carriers of the CC and CT genotypes of the SLC30A8 had higher stimulated C-peptide levels the first year after onset of type 1 diabetes compared with those with the TT genotype (most of them display ZnT8WA but not ZnT8RA) [122].

According to one study patients with multiple autoantibodies needed higher doses of exogenous insulin at 12, 18 and 24 months after clinical onset and they had lower serum C-peptide concentrations during the second year after diagnosis [19].

OGTT

Oral glucose tolerance test (OGTT) is the test preferred to validate glucose tolerance and to decide if a patient has diabetes, in uncertain cases. It is the test that best reflects the natural course of diabetes.

Despite increasing glucose (fasting, 2h glucose and AUC glucose levels, within normal range) in individuals with first or second degree relatives with type 1 diabetes, there is little change in the C-peptide measures, including fasting C-peptide, peak C-peptide and AUC C-peptide but with a trend of higher values [129]. The largest changes in the C-peptide response in OGTT is seen during the last 6 months before diagnosis. Within the same individual who is progressing to type 1 diabetes, the OGTT can vary between the normal and dysglycemic states, but once dysglycemia occurs the risk for subsequent type 1 diabetes is high. It is
possible that the glycemic fluctuations result from variability in insulin sensitivity, which could be a factor in the pathogenesis of type 1 diabetes [129].

IvGTT

Intravenous glucose tolerance test (IvGTT) is widely used in research to describe the residual β cell function, with the glucose disappearance rate - the k-value. Glucose values are plotted semi-logarithmically against time, with plasma glucose concentration depicted on the y-axis (the logarithmic scale) and the time in minutes on the x-axis. The disappearance rate of glucose is estimated on the near-straight line to calculate the k-value when the glucose level is reduced by 50%. K-values <1% per min are used to indicate diabetes while values between 1.0-1.2% are regarded as borderline [134]. The First Phase Insulin Response (FPIR) is the sum of serum insulin at the one and three minutes time points after the infusion of glucose. Reference ranges for FPIR in healthy children are poorly defined. FPIR increases with age, especially during puberty, when insulin sensitivity is decreased [83]. A decreased FPIR value is regarded as a late sign of β cell dysfunction with a high risk of type 1 diabetes development in the near future [37]. Repeated IvGTT have shown that the reproducibility of the FPIR is relatively high [83]. However, the value of FPIR in the prediction of type 1 diabetes has been questioned [47, 83]. Family studies have shown that a reduced FPIR to IvGTT is associated with an accelerated progression to type 1 diabetes, particularly in young individuals with first-degree relatives with type 1 diabetes [47, 73, 80]. FPIR values were lower at baseline in progressors and remained lower throughout the study period. The glucose elimination rate decreased over time in one study [129]. In yet another study the most important risk factor for the development of type 1 diabetes was the expression of multiple autoantibodies, especially in the presence of a low FPIR [135]. However, in a Finnish study of 1-5-year-old children, FPIR was subnormal in 42% of children analyzed with IvGTT soon after seroconversion to ICA positivity [83]. ICA greater than 20 JDFU, IAA and an increasing number of positive autoantibodies were all independent predictors of low FPIR. A number of these patients have remained non-diabetic for a relatively long time despite very low FPIRs [83]. According to another study FPIR was primarily associated with positivity for multiple autoantibodies and among these patients the IAA positive
patients had the lowest FPIR [136]. The highest risk HLA DQ 2/8 genotype is associated with low FPIR in ICA positive children according to one study, but most studies have not found any association between HLA and FPIR [83, 136].

Hence, FPIR in IvGTT and 2h glucose in OGTT demonstrate significant but moderate prognostic value for development of type 1 diabetes, whereas fasting plasma glucose does not have a predictive value [47]. However, the combination of 2-h glucose, peak C-peptide, and AUC C-peptide can improve the prognostic accuracy of OGTT. According to one study, the addition of autoantibody titers and/or IvGTT markers did not increase the prognostic accuracy further than OGTT alone [47]. Stimulated C-peptide, body mass index (BMI) and age have also been used to predict type 1 diabetes as independent contributors [129].
5. Aims and hypothesis

The specific aims of the present thesis were:

1. To test the hypothesis that autoantibodies to all three Zinc transporter 8 variants in addition to GAD65, IA-2, insulin and ICA in combination with HLA would improve the diagnostic sensitivity of childhood type 1 diabetes by detecting children who otherwise would have been autoantibody negative, as well as to determine if the different islet autoantibody analysis could replace each other.

2. To further investigate the relationships between the three ZnT8A in addition to the other islet autoantibodies as well as their association with different HLA-DQ and to create an algorithm for cost effective screening for classification of diabetes in Sweden.

3. To examine whether ICA could be identified in children with newly onset diabetes mellitus but negative for autoantibodies against GAD65, IA-2, insulin or any of the three variants of the ZnT8 and to evaluate the usefulness of a commercial ELISA (Medizym ICAscreen) by comparing it with the classical ICA-IF assay.

6. Subjects and Study Design

Sweden has 9 400 000 inhabitants. Skåne, the southern province of Sweden, has 1 200 000 inhabitants. Almost all children and adolescents (0-18 years old) diagnosed with type 1 diabetes in Sweden are cared for in 44 Pediatric Departments in the hospitals in Sweden and staff in all the hospitals are engaged in recruiting the children to studies and quality register for type 1 diabetes. Blood (serum/plasma) was collected at clinical type 1 diabetes diagnosis in Skåne children from January 1996 to April 2005 and from all children in Sweden from May 2005 and is still ongoing. The blood samples are stored at -20°C and analyzed for islet autoantibodies and HLA. The three first studies in this thesis are based on these children from either Skåne or the entire Sweden and on blood samples taken at the clinical diagnosis of type 1 diabetes. For the fourth study non-diabetic children were recruited between May 2009 and January 2012 to secondary prevention from three different studies: DiPiS - Diabetes Prediction in Skåne study, TEDDY - The Environmental Determinants of Diabetes in the Young study and TrialNet, a network of Clinical Centers in different countries for the study, prevention, and treatment of type 1 diabetes (http://www.diabetestrialnet.org/). These children are followed in the different studies because of an increased risk for type 1 diabetes, based on HLA, islet autoantibodies and/or heredity. For this current study the inclusion criteria were that the child should have GADA and at least one other islet autoantibody. Islet autoantibodies (not ICA) and HLA genotyping was performed and the children were further analyzed with both OGTT and IvGTT to evaluate glucose tolerance and metabolism.

In paper I serum samples at diagnosis before the start of insulin treatment were available from 686 consecutive patients who were classified with type 1 diabetes according to the recommendation by ADA [8]. The serum samples were analyzed for islet autoantibodies (ICA, GADA, IA-2A, IAA, ZnT8RA, ZnT8WA and ZnT8QA) and HLA genotyping was performed. There were 373 (54%) boys and 313 (46%) girls in the study. The mean age of the children at type 1 diabetes diagnosis was 9.8 years.

In paper II we analyzed patients from the Better Diabetes Diagnosis (BDD) study, a nationwide prospective study for newly diagnosed diabetes patients who are younger than 18 years old. For this study we were only interested in the patients
with type 1 diabetes. Blood samples at diagnosis, before insulin or within three days after diagnosis and start of treatment, were analyzed for islet autoantibodies (GADA, IA-2A, IAA, ZnT8RA, ZnT8WA and ZnT8QA) and were HLA genotyped. Between May 2005 and August 2010 a total of 3719 diabetes patients were analyzed in the BDD study and 3165 (56% boys) were classified as type 1 diabetes according to ADA recommendations [8] while 58 were classified as type 2 diabetes, 33 as maturity onset diabetes of the young (MODY), 9 as secondary diabetes mellitus and 326 as unclassifiable type of diabetes mellitus at the time of clinical diagnosis (Figure 3). Only 128 patients were excluded because of missing data or serum. The group of 326 unclassifiable type of diabetes likely includes patients with type 1 diabetes but was excluded from this study as the final classification was missing. The median age of the children at type 1 diabetes diagnosis was 10.1 years (range 0-17.9 years) (10.6 for boys, 9.7 for girls).
Figure 3. Flow chart of the BDD study (Paper II and Paper III).
Paper III is based on the BDD patients from the same study period as paper II. The analyses of islet autoantibodies and HLA typing in Paper II were performed soon after diagnosis but with the results from paper II we were interested in the analysis of ICA in all islet autoantibody negative patients, including all types of diabetes except neonatal diabetes (n=4) and secondary diabetes (n=37). The number of patients in Paper III was higher than in paper II because the BDD study is continuously updated with information from the Pediatric departments and from SWEDIABKIDS [13]. Patients with known MODY, type 2 and type 1 diabetes were analyzed. At the time of the ICA analysis we had diagnostic information from 3708 children and adolescents aged 0-18 years with a median age of 10.4 years and 56% boys [137]. Only GADA and IA-2A were analyzed in 122 children due to limited amount of specimen, leaving 3545 samples with full evaluation of GADA, IA-2A, IAA and the three ZnT8A. A total of 341 children were found to be negative for all these six autoantibodies, the median age was 12.8 years, and 60% were boys. Among these 341 children 53 had borderline levels of the different autoantibodies but the other 288 were islet autoantibody negative. Furthermore, 203 out of the 288 autoantibody negative children were classified with type 1 diabetes. In Figure 3 we have added the ICA-positive patients in the otherwise autoantibody negative group from paper II.

In paper IV we analyzed baseline data from 47 non-diabetic children with a genetic risk of type 1 diabetes, GADA and at least one more islet autoantibody from the Diabetes Prevention - Immune Tolerance (DiAPREV-IT) study, a study designed to evaluate immune tolerance with Alum-GAD (Diamyd®). Children aged 4-18 years were recruited to this study from DiPiS, TEDDY and TrailNet and 54 children were screened before we had 50 children that met the inclusion criteria. Three of these children were not included in this paper as they were not pre-pubertal and outliers in age and hence had different reference values for the glucose metabolism evaluation. The median age of the 47 children in this study was 5.1 years (range 4.0-9.2 years) and 25 were boys and 22 were girls.
7. Methods

Autoantibody analyses

ICA

Autoantibodies to islet cell cytoplasm (ICA) in paper I were determined in a two-colour indirect immunofluorescence assay (ICA-IF) performed on sections of frozen human pancreas, as described previously [138]. Our laboratory participated in the 13th Immunology of Diabetes workshop standardization and showed a sensitivity and a specificity of 100% [90]. Levels of ICA were expressed in JDF-U, using the world reference standard curve based on the international JDF reference sera sample.

In paper III islet autoantibody negative (GADA, IA-2A, IAA, ZnT8A) diabetes patients were screened with a commercial ELISA (Medizym ICAscreen; ref 3804, Medipan Gmbh, Berlin, Germany) and if deemed positive by this assay the samples were re-analyzed with classical ICA-IF and if positive in the initial dilutions (1:2, 1:8 and 1:64) the samples were titrated to a final dilution. If the sample was negative in these three dilutions it was considered negative. The Medizym ICAscreen assay was carried out according to the instructions from the manufacturer and fluorescence was read at 450 nm (reference wavelength 630 nm) (MicroReader 4Plus, 4028-052, Hyperion Inc, Miami, FL, USA). In order to validate this assay we used a separate set of 100 children newly diagnosed with type 1 diabetes mellitus (median age 10 yrs range 1-15; M/F=1.4) and a set of 199 healthy blood donors (median age 43 yrs; range 19-65; M/F=1.3). Using these healthy controls, the cut-off for a positive value was defined as binding index above 1.5 (97.5% percentile). The sensitivity was 90% and the specificity 97.5%. In 97% of all measurements the coefficient of variation (CV) for duplicates was less than 10%. The inter assay variation for a positive control serum (mean binding index 5.9 ± 0.63) was 11% (n=47) and the intra assay variation for the same control was 2.6% (n=24). The samples from the 100 children were also analyzed with ICA-IF which was determined with a prolonged two colour immunofluorescence assay using human pancreas as substrate, previously
described in detail [139]. The samples were diluted 1:2, 1:4, 1:8 etc until negative, and the highest positive titer was converted into JDF-U according to a standard curve for this specific pancreas. The lower detection limit was 3 JDF-U.

ICA was not analyzed in paper II and IV.

GADA

Autoantibodies to glutamic acid decarboxylase (GADA) in paper I were analyzed using a commercially available kit for radioligand binding assay (RBA) of GAD65 autoantibodies according to the instructions by the manufacturer (RSR Limited, Cardiff, UK). The RSR kit has been validated in the Diabetes Autoantibody Standardization Program (DASP) with 74% study sensitivity and 96% study specificity. The CV was 8.9% at level 2.0 Units/mL and 14.2% at level 44.6 units/mL.

In paper II to IV GAD65 and IA-2 autoantibodies were analyzed together. Recombinant GAD65 and IA-2 were labeled with 35S-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 [95]. 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 (22). GADA and IA-2A were analyzed in a RBA [95] in samples eluted from dried blood spots (DBS). 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 35S-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 35S-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, Boston, MA, USA) was added and the radioactivity of antibody bound 35S-labelled GAD65 or IA-2 was counted in a Wallac Microbeta Trilux (Perkin Elmer) beta counter. GADA and IA-2A levels were expressed as units per mL (U/mL) derived from the WHO standard 97/550 [90]. Samples were considered positive if GADA 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 had a workshop sensitivity of 68 % and specificity 99% for GADA and a 60% workshop sensitivity and 99% specificity for IA-2A [140].

**IA-2A**

In paper II to IV IA-2A was analyzed with the combined analysis described above.

In paper I autoantibodies to islet-antigen-2 (IA-2A) were analyzed in a RBA using a kit analogous to the GADA kit according to the instructions by the manufacturer (RSR Limited, Cardiff, UK). The RSR kit has been validated in DASP with 68% study sensitivity and 100% study specificity. The CV was 7.7% at level 2.6 Units/mL and 5.8% at level 25.6 Units/ml.

**IAA**

In paper I, II and IV insulin autoantibodies (IAA) were determined in a non-competitive RBA using 125I-insulin essentially as described [141] and modified [142]. The results were expressed in arbitrary units derived from in-house positive and negative standard samples. Intra-assay CV was 6% and inter-assay CV 13%. Our laboratory has been validated in DASP 2010 with 26% study sensitivity and 100% study specificity [140].

**ZnT8A**

In all four papers autoantibodies to all three Zinc transporter variants were analyzed in RBA. The ZnT8R, ZnT8W and ZnT8Q were performed separately with 5 μL of human sera essentially as described [101]. Duplicate serum samples
were incubated over night at +4°C with labeled antigen diluted in antigen buffer. Antibody-bound was separated from free antigen by Protein A-Sepharose (Zymed, San Francisco, CA, USA). Bound radioactivity was converted into in-house units using a high-titer standard with high ZnT8RA, ZnT8WA or ZnT8QA reactivity and the results were expressed in arbitrary units derived from these in-house positive and negative standard samples. Intra-assay CV for the ZnT8RA was 6%, ZnT8WA 5% and ZnT8QA 4%, respectively. Inter-assay CV for ZnT8RA was 7%, ZnT8WA 8% and ZnT8QA 10%, respectively. Our laboratory has been validated in DASP in 2011 with 50% study sensitivity and 100% study specificity for ZnT8RA, 46% study sensitivity and 100% study specificity for ZnT8WA and 38% study sensitivity and 100% study specificity for ZnT8QA [143].

**HLA**

In all papers HLA-DQB1 and DQA1 genotypes were typed by sequence-specific oligonucleotide probes on DBS used directly for polymerase chain reaction (PCR) amplification of DQA1 and DQB1 alleles as described [40] using a DELFIA Hybridization assay (Perkin Elmer, Boston, MA, USA). 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 define the DQA1*0201, 03 and 05 alleles. The HLA frequencies of the patients in Paper I and II were compared with 2000 newborn children in the DiPiS study [144-145].

**BMI**

In paper IV body mass index (BMI) was measured and the standard deviation (BMI SD) was used for the analysis. The Swedish mean ± 1, 2, 3 SD reference ranges of BMI were used [146]. These are also based on international surveys [147]. The child’s height and weight was measured and the BMI SD was plotted on the Swedish curves.
Plasma glucose

In paper IV venous plasma glucose was determined in a bedside HemoCue® (Ängelholm, Sweden) glucometer.

HbA1c

HbA1c in paper IV was measured at Skåne University Hospital, SUS, Laboratory Medicine in Malmö, using the accredited IFCC VARIANT™ TURBO HemoglobinA1c Kit- 2.0 program (Bio-Rad Laboratories, Hercules, CA, USA) using 27-42 mmol/mol as the normal reference range.

C-peptide and insulin

In paper IV plasma C-peptide and plasma insulin was measured at Skåne University Hospital, SUS, Laboratory Medicine in Malmö using accredited electrochemiluminiscence immunoassays (Siemens AG, Munich, Germany).

OGTT

In paper IV OGTT (120 minutes) was performed in all participants except one who refused. After fasting overnight, Nutrical® (N.V. Nutricia Zoetermeer, Holland) was given in a dose of 1.75 g glucose per kg body weight. All participating children drank the solution within 5 minutes. Venous plasma glucose, serum C-peptide and insulin were measured at 0, 30, 60, 90 and 120 minutes.
Plasma glucose values ≥11.1 mmol/L after two hours were used for type 1 diabetes diagnosis, values ≤7.8 mmol/L were considered normal and values between 7.8 mmol/L and 11.0 mmol/L were considered as impaired glucose tolerance according to the ADA recommendations [8].

IvGTT

In paper IV IvGTT (90 minutes) was performed in all participants. After fasting overnight (since 24.00 hrs), glucose (500 mg/kg body weight) was injected within 3 minutes as a 30% solution. Venous plasma glucose, serum C-peptide and insulin were measured at -10, 0, 1, 3, 5, 7, 10, 30, 50, 70 and 90 minutes and glucose values plotted semi-logarithmically against time. The disappearance rate of glucose was estimated on the near-straight line to calculate the k-value. K-values <1.0 were used to indicate diabetes while values between 1.0-1.2 were regarded as borderline [134]. FPIR is the sum of serum insulin at the one and three minutes time points after the infusion of glucose. FPIR values of ≤ 30 µU/mL insulin were considered as a marker of reduced β-cell function in this prepubertal group [134].

Impaired glucose metabolism

In paper IV impaired glucose metabolism was defined as either a k-value of <1.2, a FPIR value of ≤30 µU/mL insulin or a plasma glucose ≥7.8 but ≤ 11.1 mmol/L at 120 min in the OGTT.
Sample repository

The analyses in the present thesis are dependent on serum samples that have been stored in Biobank Region Skåne, BD31. As described earlier, these samples have been collected from children at the Department of Pediatrics in Sweden, since 1996. Whether the results of our study might have been influenced by the extended storage of some of the serum samples at -20°C or -80°C remains an issue.

Statistics

In paper I and II statistical analyses were performed using SPSS statistical software (version 17.0 and 18.0 respectively; SPSS, Chicago, IL, USA). Differences in proportions between groups were tested using the χ² test or Fisher’s exact test when appropriate. Bonferroni correction was used to correct for the number of comparisons. Odds ratios (OR) with 95% confidence interval (CI) were calculated from simple logistic regression models to evaluate the degree of association between the categorical variables. P-values < 0.05 were considered significant.

To measure the agreement between islet autoantibodies we used the kappa statistic [148]. A kappa value of one indicates perfect agreement, whereas a kappa of zero is what would be expected by chance and negative values indicate a systematic disagreement between the observers [148].

Interpretation of kappa:
Kappa Agreement
<0 Less than chance agreement
0.01-0.20 Slight agreement
0.21-0.40 Fair agreement
0.41-0.60 Moderate agreement
0.61-0.80 Substantial agreement
0.81-0.99 Almost perfect agreement
Kappa can quantify the magnitude of agreement between observers with a numerical rating of the degree to which they agree. The calculation is based on the difference between the observed agreement and the expected agreement due to chance alone. The estimated kappa itself can also be due to chance. P value in kappa tests whether the estimated kappa is due to chance or not. It does not test the strength of the agreement. P values and confidence intervals are sensitive to sample size and a kappa above zero will become statistically significant with a large enough sample size, but the kappa may not be reliable for rare observations [148].

In paper III and IV the levels of the different parameters are expressed as median and range since not normally distributed. In paper III SPSS Statistical software (version 20; SPSS, Chicago, IL, USA) was used for the statistical calculations. Coefficient of variation (CV) for duplicate samples was calculated as the ratio between standard deviation and mean value. Spearman rank correlation test was used to test for correlation between indexes from ICA-ELISA and levels of ICA from ICA-IF (JDF-U).

In paper IV differences in proportions between groups were tested using χ² test or Fisher’s exact test when appropriate. Bonferroni correction was used to correct for the number of comparisons. Mann Whitney U test was used when comparing the two groups with normal and impaired glucose metabolism. P-values <0.05 were considered significant.

Ethical aspects

The Human Research Ethics Committee of the Faculty of Medicine, Lund University, Lund, Sweden approved the Skåne study. The Regional Ethical Review Board in Stockholm, Sweden, approved of the BDD study and the Regional Ethical Review Board in Lund, Sweden, as well as the Swedish Medical Products Agency approved of the DiAPREV-IT study.
8. Results and Discussion

The results from Paper I and paper II are presented together. In both these papers we studied autoantibodies and HLA alleles, haplotypes and genotypes in children and adolescents at the time of type 1 diabetes diagnosis. A total of 686 patients from Skåne, aged 1.0 to 18.8 years and diagnosed between January 1996 and April 2005, were studied in Paper I. In Paper II 3165 patients from Sweden, aged 0.1 to 17.9 years and diagnosed between May 2005 and August 2010, were studied. The aims of both studies were to test the hypothesis that autoantibodies to all three ZnT8 (ZnT8RA, ZnT8WA, ZnT8QA) variants in addition to previously known autoantibodies, and in combination with HLA, would improve the diagnostic sensitivity of childhood type 1 diabetes. We also wanted to determine if the different islet autoantibody analyses could replace each other, and finally to create an algorithm for cost effective screening for classification of diabetes in Sweden.

Ranking and frequency of autoantibodies

In Paper I we found ICA to be the most common islet autoantibody at onset of childhood type 1 diabetes, which is consistent with studies in Finnish children [84]. IA-2A was the second most common autoantibody. In paper II IA-2A was the most common autoantibody as ICA was not analyzed, followed by GADA in both studies. In Paper I ZnT8WA was found in 4 more patients than ZnT8RA (both 50%), but in Paper II ZnT8RA was more common than ZnT8WA. The least common autoantibodies in both studies were IAA followed by ZnT8QA (Figure 4). The ranking order of IA-2A, GADA and IAA is consistent with previous studies but we could reveal that both ZnT8RA and ZnT8WA were more common than IAA [52, 105]. Paper I is the first published ranking including the three ZnT8A in children and adolescents at the time of diagnosis. The prevalence of the different autoantibodies was comparable between the two studies and also with the results from the first publication with all three ZnT8A [125].
When ZnT8A was considered as one group, 65% were positive for one, two or all three of the ZnT8A in both studies. The ZnT8A contributed to the diagnostic sensitivity of type 1 diabetes as 2-3.4% of the patients in the studies had one, two or all three ZnT8A without having any other islet autoantibody [98, 120]. The high frequency of children positive for ZnT8A is comparable to previous reports [98]. Triple ZnT8A were most common and double ZnT8A were least common among the ZnT8A positive patients in both studies (Figure 5). The ZnT8QA was not found as the only autoantibody in paper II and only one patient in paper I displayed ZnT8QA as a single positive autoantibody. ZnT8QA was rare among patients displaying three or less of all the autoantibodies in both studies.
Figure 5. Venn diagram analysis showing the distribution of the ZnT8RA, ZnT8WA and ZnT8QA among the 449 ZnT8A-positive type 1 diabetes patients in paper I.
When we analyzed the patients displaying only one autoantibody, the results in the two studies differed. In Paper I GADA was most common, followed by ICA, IAA, IA-2A, ZnT8WA, ZnT8RA and ZnT8QA. In Paper II IA-2A was most common, followed by GADA, ZnT8RA, IAA and ZnT8WA. Based on the results from both studies we suggest that ZnT8QA is the least cost efficient islet autoantibody to analyze to improve diagnostic sensitivity. However, we propose that ZnT8QA is useful for prediction as it can be the only ZnT8A in combination with the other islet autoantibodies.

Number of autoantibodies

The number of islet autoantibodies was next analyzed (Figure 6). A total of 6% and 10% of the patients, in Paper I and Paper II respectively, of the patients had all autoantibodies and only 5% and 7%, respectively, were islet autoantibody-negative. With the inclusion of ZnT8A, the diagnostic sensitivity of islet autoantibodies for type 1 diabetes increased from 93% to 95% in Paper I and from 90% to 93% in Paper II. The reduction in autoantibody negative patients with the addition of the ZnT8A was 28% and 33%, respectively, compared to 26% in another study [98]. The ZnT8A were more common among the children that displayed two or all three of the GADA, IA-2A or IAA than among those displaying only one of these autoantibodies.
Autoantibodies and their relation to age

Both in Paper I and Paper II we analyzed if there was a relationship between the different autoantibodies and the age of the patient at diagnosis, as well as between the number of autoantibodies and the age of the patient. According to several studies [19, 52, 92], IAA is more frequent and ZnT8A is less frequent among young children [115], which we could confirm (Figure 7). IAA was found significantly more often in children below five years of age (81%) compared to those who developed type 1 diabetes above 15 years of age (26%) in Paper I (Figure 7). This finding was confirmed in Paper II.
We also confirmed that all three ZnT8A are less frequent in the youngest children. We found a difference in frequency between children younger than both two and five years compared with those older than two and five years, respectively, for ZnT8RA, ZnT8WA as well as ZnT8QA (Figure 3 in Paper II). However, the combined ZnT8A were almost as common in the young as in the older children (Figure 1 in Paper I), which further supports the importance of measuring all three ZnT8A. Among the other islet autoantibodies there was no indication that the frequency was related to the age at diagnosis in Paper I (Figure 7), but in Paper II we found a significant difference (p<0.0001) for GADA, which was less frequent in the group of children younger than 10 years of age compared to the children older than 10 years of age.

Multiple autoantibodies were common at all ages, but the number of patients diagnosed without any autoantibody increased with increasing age at onset (p-value for trend 0.009 in Paper I). However, among the youngest children (<2 years) the frequencies could vary due to the smaller number of patients. Children displaying all autoantibodies showed similar frequencies as the islet autoantibody negative patients in both studies (Figure 8).
Figure 8. The number of autoantibodies in patients in % in relation to age at diagnosis in different age groups, age groups year by year in Paper II.

It cannot be excluded that the autoantibody negative patients have had auto antibodies that disappeared before the clinical onset. This is consistent with ongoing longitudinal studies of children at risk, indicating that any of the islet auto antibodies may appear to reach significant titers, which drop to negativity at any point during the preclinical period. IAA would be the autoantibody that may disappear most often. There is also a possibility that some of the islet autoantibody negative children had an islet autoantibody in the cord blood [71, 149]. It was previously reported that patients with islet autoantibodies in their cord blood may be autoantibody negative at diagnosis [71]. Furthermore, there is the possibility that some of the islet autoantibody negative patients do not have type 1 diabetes or that they may have autoantibodies to less frequent autoantigens [127]. We observed that 1.5% of the patients in paper I were positive for ICA alone, which indicates that the search for additional autoantigens should continue. The autoantibody negative patients in paper II were further analyzed for ICA in paper III.
Autoantibodies and gender

We next analyzed if there were any gender differences. As expected, the data in both paper I and paper II, showed a higher prevalence of boys (54% and 56%, respectively). In Paper I we found significant differences in gender frequencies between different age groups but the number of patients in each group was small (Figure 9). There were also more boys among the patients with all autoantibodies and among the ZnT8QA and ZnT8WA positive patients in Paper I. However, we could not find the same gender differences in Paper II and we suggest that the results from Paper I could be due to chance. GADA has previously been described as more common among girls [19, 52, 92]. We could confirm this in Paper I but not in Paper II.

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>Age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18</td>
</tr>
<tr>
<td>Boys</td>
<td>1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18</td>
</tr>
<tr>
<td>Girls</td>
<td>1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18</td>
</tr>
</tbody>
</table>

Figure 9. Number of patients in total and divided by sex in Paper I, showing significant gender differences among the youngest children.
Can any of the autoantibody analyses replace another analysis?

As the analysis of ICA is complicated and expensive, we tested whether the ZnT8A might replace ICA as a type 1 diabetes marker in Paper I. ICA together with ZnT8A was found in 392 (57%) patients, whereas 141 (21%) patients had ICA but not ZnT8A, 57 (8%) patients had ZnT8A but not ICA and 96 (14%) patients did not have either ICA or ZnT8A. According to this distribution ZnT8A cannot replace the ICA analysis.

We next analyzed the combinations of all islet autoantibodies. Among the multiple (≥ 2) autoantibody positive patients we found a strong association between all three variants of ZnT8A. In Paper II ZnT8RA and ZnT8QA were most strongly associated (OR 43; p<0.0001) followed by ZnT8WA and ZnT8QA (OR 40; p<0.0001). All three ZnT8A were more strongly associated with IA-2A (OR IA-2A with ZnT8RA 3.2, with ZnT8WA 3.6 and with ZnT8QA 3.0; p<0.0001 for all) than with GADA and IAA (Table I in paper II).

To evaluate whether some islet autoantibody combinations are more frequent than others and if one analysis could replace another, we used kappa statistics (Table II in Paper I), because it takes into account that observations can agree or disagree simply by chance [148]. In Paper I we presented the results from the ZnT8A as one group since there were no differences when analyzing the three ZnT8A separately in the kappa analysis. We did not find any substantial or almost perfect agreements between the different islet autoantibody tests, which supports the idea that no autoantibody analysis can replace another. There was a moderate or fair agreement between ICA and all autoantibody combinations except IAA, which underscores the importance of measuring IAA. Kappa statistics in paper II were done with the three ZnT8A separately to be able to decide if the different ZnT8A analyses could replace each other (Table 1).
Table 1. Agreement of pairs of islet autoantibodies to mark islet autoimmunity in newly diagnosed type 1 diabetes children. Kappa statistics in Paper II.

<table>
<thead>
<tr>
<th>Antibody1 – Antibody2</th>
<th>Rank</th>
<th>kappa (SE)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZnT8WA – ZnT8QA</td>
<td>1</td>
<td>0.60 (0.01)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ZnT8RA – ZnT8QA</td>
<td>2</td>
<td>0.51 (0.01)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ZnT8RA – ZnT8WA</td>
<td>3</td>
<td>0.42 (0.02)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ZnT8RA – IA-2A</td>
<td>4</td>
<td>0.23 (0.02)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ZnT8WA – IA-2A</td>
<td>5</td>
<td>0.23 (0.02)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ZnT8QA – IA-2A</td>
<td>6</td>
<td>0.14 (0.01)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ZnT8RA – GADA</td>
<td>7</td>
<td>0.09 (0.02)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IAA – IA-2A</td>
<td>8</td>
<td>0.09 (0.01)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IAA – GADA</td>
<td>9</td>
<td>0.08 (0.02)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ZnT8WA – GADA</td>
<td>10</td>
<td>0.06 (0.02)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ZnT8WA – IAA</td>
<td>11</td>
<td>0.06 (0.02)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>ZnT8QA – IAA</td>
<td>12</td>
<td>0.06 (0.02)</td>
<td>0.001</td>
</tr>
<tr>
<td>ZnT8RA – IAA</td>
<td>13</td>
<td>0.05 (0.02)</td>
<td>0.004</td>
</tr>
<tr>
<td>ZnT8QA – GADA</td>
<td>14</td>
<td>0.05 (0.02)</td>
<td>0.001</td>
</tr>
<tr>
<td>IA-2A – GADA</td>
<td>15</td>
<td>0.02 (0.02)</td>
<td>0.153</td>
</tr>
</tbody>
</table>

*a = ranked by agreement to mark autoimmunity (kappa).*
We confirmed that all three ZnT8A are common together with each other (as proven by the OR-calculation above), but that the analyses of the different variants of ZnT8A cannot replace each other since the kappa value is not close to one. It also confirms that there is a fair agreement between IA-2A and ZnT8RA or ZnT8WA but only a slight agreement between all the other autoantibody combinations as in Paper I.

**HLA risk genotypes**

All patients were typed for HLA-DQ alleles known to be associated with type 1 diabetes and compared with the HLA frequency in controls from the DiPiS study representing the general population in the county of Skåne. The associations between HLA-DQ genotypes and type 1 diabetes in Paper II are summarized in Table 2, also showing the number of islet autoantibodies in each genotype. Type 1 diabetes was significantly associated with six different HLA-DQ genotypes, all containing DQ8, DQ2, or both (DQ2/8; 32% in Paper I and 30% in Paper II). Two thirds of the patients had one of these six HLA-DQ genotypes. DQ8 was the most common haplotype, three times as common in the patients (40%) compared to the controls (13%) (OR 4.5 and 5.4 in Paper I and Paper II respectively). DQ2 was twice as common (28% and 26%, respectively) among the patients compared to the controls (13%) (OR 2.6 and 2.4, respectively). At least one of these two haplotypes was found in 87% and 86%, respectively, of the patients compared to 45% among the controls (OR 6.8 and 9.0, respectively).
Table 2. HLA-DQ genotypes, the number of islet autoantibodies and OR, 95th CI at diagnosis of type 1 diabetes in 3165 children in Paper II compared to 2000 children genotyped from the general population in the DiPiS study. Genotypes with OR that were not significant or negative were collapsed into groups according to the DQ2 and DQ8 haplotypes.

<table>
<thead>
<tr>
<th>Haplotype 1</th>
<th>Haplotype 2</th>
<th>Number of islet autoantibodies</th>
<th>Total</th>
<th>BDD%</th>
<th>DIPI n</th>
<th>DIPI %</th>
<th>OR</th>
<th>CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DQA1-B1</td>
<td>DQA1-B1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>05-02</td>
<td>03-0302</td>
<td>62</td>
<td>153</td>
<td>179</td>
<td>200</td>
<td>151</td>
<td>140</td>
<td>59</td>
<td>944</td>
</tr>
<tr>
<td>X-0302</td>
<td>X-0302</td>
<td>12</td>
<td>38</td>
<td>57</td>
<td>65</td>
<td>76</td>
<td>67</td>
<td>40</td>
<td>355</td>
</tr>
<tr>
<td>X-0302</td>
<td>X-0604</td>
<td>5</td>
<td>16</td>
<td>31</td>
<td>24</td>
<td>39</td>
<td>34</td>
<td>8</td>
<td>157</td>
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<td>X-0601</td>
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<td>62</td>
<td>35</td>
<td>9</td>
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<td>25</td>
<td>48</td>
<td>22</td>
<td>20</td>
<td>21</td>
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<td>154</td>
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<td>33</td>
<td>20</td>
<td>23</td>
<td>22</td>
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<td>155</td>
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<tr>
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<td>7</td>
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<td>18</td>
<td>19</td>
<td>15</td>
<td>17</td>
<td>6</td>
<td>99</td>
</tr>
<tr>
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<td>13</td>
<td>12</td>
<td>22</td>
<td>12</td>
<td>1</td>
<td>75</td>
</tr>
<tr>
<td>05-02</td>
<td>X-X</td>
<td>28</td>
<td>58</td>
<td>74</td>
<td>53</td>
<td>41</td>
<td>42</td>
<td>6</td>
<td>302</td>
</tr>
<tr>
<td>X-0302</td>
<td>X-X</td>
<td>13</td>
<td>37</td>
<td>57</td>
<td>53</td>
<td>63</td>
<td>52</td>
<td>26</td>
<td>301</td>
</tr>
<tr>
<td>X-X</td>
<td>X-X</td>
<td>41</td>
<td>42</td>
<td>58</td>
<td>62</td>
<td>50</td>
<td>47</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2000</td>
</tr>
</tbody>
</table>

Number of islet autoantibodies

HLA and autoantibodies

Previous studies have shown a positive association between DQ8 and ICA, IA-2A and IAA and between DQ2 and GADA but a negative association between DQ2 and IA-2A as well as IAA [29, 45, 52, 73, 84, 92, 96, 99]. In Paper I we could confirm the positive associations between DQ8 and ICA (p=0.005) as well as with IA-2A (p<0.0001) and between DQ2 and GADA (p=0.03<0.0001). We could also confirm the negative association between DQ2 and IA-2A (p<0.0001) as well as with IAA (p=0.0005). In Paper I DQ8 was positively associated with IAA (p=0.003), but in Paper II IAA was not associated with DQ8/8 and 8/X, where X is not 2 (p=0.7), but with DQ2/8 (p=0.002). In Paper II we also found that GADA was positively associated with DQ2/8 (p=0.006) and negatively associated with DQ8 (p<0.0001).

We discovered that the combined ZnT8A (p=0.0005) as well as the individual ZnT8RA (p=0.002) and ZnT8WA (p=0.01) were associated with DQ B1*X-0604 (DQ6.4) in Paper I. In this study we also found an association between DQ6.4 and
both GADA (p=0.03) and IAA (p=0.03) which could not be confirmed in Paper II. Based on the large number of patients in Paper II, we decided to analyze the haplotypes and genotypes more selectively to exclude the interaction from DQ2 and DQ8 (Table 3). We confirmed that all three ZnT8A were associated with the DQ6.4 haplotype but also with the DQ8 haplotype. We could extend this observation and demonstrate that the DQ6.4/6.4 and 6.4/X (X is not DQ2 or DQ8) genotypes as well as the DQ 8/8 and 8/X (X is not DQ2) genotypes were all positively associated with each one of the three ZnT8A. In contrast, a negative association between DQ2/2 and 2/X (X is not DQ8) was observed for ZnT8WA and ZnT8QA but not for ZnT8RA. All three ZnT8A were also negatively associated with the DQ2/8 genotype (Table 3).

Table 3. Associations between HLA-DQ genotypes and islet autoantibodies (OR and p-value) at diagnosis of type 1 diabetes in 3165 children in Paper II.

<table>
<thead>
<tr>
<th>ZnT8RA</th>
<th>ZnT8WA</th>
<th>ZnT8QA</th>
<th>GADA</th>
<th>IA2A</th>
<th>IAA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.7</td>
<td>0.7</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p&lt;0.001</td>
<td>p=0.003</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.3</td>
<td>0.7</td>
<td>p=0.0005</td>
</tr>
<tr>
<td>DQ 2/2 and 2/X</td>
<td></td>
<td></td>
<td>1.2</td>
<td>1.4</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p=0.003</td>
<td>p&lt;0.001</td>
<td>p=0.0001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.6</td>
<td>2.3</td>
<td>n.s.</td>
</tr>
<tr>
<td>DQ 8/8 and 8/X</td>
<td></td>
<td></td>
<td>0.8</td>
<td>0.9</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p=0.0045</td>
<td>p=0.049</td>
<td>p=0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.6</td>
<td>2.2</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p=0.006</td>
<td>p&lt;0.001</td>
<td>n.s.</td>
</tr>
<tr>
<td>DQ 2/8</td>
<td></td>
<td></td>
<td>1.3</td>
<td>1.2</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p=0.006</td>
<td>p=0.006</td>
<td>p=0.002</td>
</tr>
<tr>
<td>DQ 6.4/6.4 and 6.4/X</td>
<td></td>
<td></td>
<td>2.0</td>
<td>2.5</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p=0.01</td>
<td>p=0.009</td>
<td>p=0.03</td>
</tr>
</tbody>
</table>

Red color represents a positive association. Blue color represents a negative association.

The fact that DQ8, but not DQ2 nor DQ2/8, is positively associated with the three ZnT8A suggests that the DQ8 heterodimer is important for ZnT8 antigen presentation. DQ6.4 is only associated with a high risk of developing type 1 diabetes together with DQ8 and DQ2 according to the OR in Table 3. Our data therefore suggest that the association between HLA-DQ 6.4/8 and 6.4/2 and type 1 diabetes is secondary to a primary association between DQ6.4 and all ZnT8A,
regardless of the polymorphic amino acid at position 325. We suggest that the negative association, i.e. inhibition of ZnT8 autoantibody formation, dominates over the DQ8 mediated antigen presentation as the heterozygous DQ2/8 patients were negatively associated with all three ZnT8A variants. Furthermore, the negative association with DQ2/8 may also explain the low frequency of ZnT8A in the very young, as the DQ2/8 genotype dominates in this age group.

A further observation was that patients without islet autoantibodies more often had neutral or low risk HLA in both studies (49% and 38%, respectively). These results further support the importance of HLA class II heterodimers to initiate the immune response to islet autoantigens. Additionally, the number of islet autoantibodies (2-5 autoantibodies) was significantly associated with DQ8 (p-value for trend <0.001) but not with DQ2 (p-value for trend 0.20) in Paper I (Table IV in Paper I). This is important as multiple autoantibodies represent the best predictor for the clinical onset of type 1 diabetes [16, 29, 76, 81, 86, 150].

**HLA and the relation to age**

Finally, we analyzed the frequency of DQ8 and DQ2 in relation to the age at diagnosis in Paper I (Figure 10). We found that DQ8 varied between 48-85% with a mean of 70%. DQ2 was more common among the children younger than five years of age but decreased thereafter. The high risk DQ2/8 genotype was most common (56%) at two years of age and least common (19%) at 16 years of age (p-value for trend <0.001). Type 1 diabetes in the absence of both DQ8 and DQ2 is uncommon but more common among the older children (p-value for trend<0.001).
Figure 10. Frequency (%) of patients in relation to age in 686 newly diagnosed Type 1 diabetes patients in Paper I.

Islet autoantibody negative patients

In paper III we wanted to see to what extent ICA was displayed in otherwise autoantibody negative children and adolescents at type 1 diabetes diagnosis, despite the classification of diabetes. At the same time we wanted to evaluate the commercial ELISA Medizym ICAscreen assay. Again we used the BDD study and hence could include all the autoantibody negative type 1 diabetes patients from paper II. Patients with neonatal diabetes and secondary diabetes were excluded from this study, leaving 3545 patients. Among these patients 341 (10%) were islet autoantibody negative. Most of these patients were considered having type 1 diabetes but some might have or do have type 2 diabetes or MODY.
ICA in islet autoantibody negative children and adolescents at type 1 diabetes diagnosis

ICA was initially analyzed with the Medizym ICA screen assay, except for one patient because of a limited amount of blood. A total of 80/340 (23%) samples were positive in the Medizym ICAscreen assay and all of these positive samples together with the only sample with a limited amount of blood, were further analyzed with the classical ICA-IF. With the ICA-IF assay 17 (5%) patients were positive for ICA. The median level of ICA in the positive patients was 30 JDF-U (range 3-183). Hence, 16 samples were positive both in the Medizym ICAscreen assay and in the classical ICA-IF assay. With the ICA analysis we only increased the islet autoantibody positive patients in the BDD study from 90%, when analyzed only for GADA, IA-2A, IAA and the three ZnT8A, to 91%.

Do patients positive for ICA display borderline levels of the other autoantibodies?

To evaluate whether the ICA positive patients more often displayed borderline levels of the other autoantibodies we studied a subpopulation of 53 of the 341 islet autoantibody negative patients with borderline levels of GADA, IA-2A, IAA and/or any of the ZnT8A. IA-2A and GADA were the most common autoantibodies with borderline levels in this subpopulation (n=22 and 21 respectively). Among the 17 ICA positive patients, seven (41%) had borderline levels of other islet autoantibodies. Two of the patients had borderline levels of two autoantibodies (GADA - IA-2A and GADA - ZnT8WA). Three of the patients had borderline levels of IA-2A and two patients had borderline levels of GADA. The levels of ICA in the patients with borderline levels of the additional autoantibodies had a lower median (19 JDF-U) and range (3-74).
Do the ICA positive patients have type 1 diabetes?

Excluding the 53 patients with borderline levels of GADA, IA-2A, IAA or ZnT8A left 288 islet autoantibody negative patients and 10 of these were positive for ICA with a median of 38.5 JDF-U and a range of 3 to 183 JDF-U. Looking at the classification of these 288 autoantibody negative patients 85 were known to have either type 2 diabetes or MODY, but none of these were positive for ICA. Hence, we had 203 patients with type 1 diabetes analyzed for ICA. Only 10/203 (5%) of the islet autoantibody negative patients without borderline levels of any islet autoantibody showed an isolated positivity for ICA.

Since all pediatric departments participating in the BDD study annually report if there has been a change in the classification of patients, we were able to include reclassification during follow-up in the analyses of the results. All but one of the 7 ICA positive patients with borderline levels of the other autoantibodies were classified as having type 1 diabetes, while the classification in one patient was uncertain. Hence, 16 of the 216 (7%) autoantibody negative patients in Paper II were ICA positive (Figure 3).

The isolated positivity for ICA despite negativity for GADA, IA-2A, IAA and ZnT8A, suggests that still other unidentified autoantigens contribute to the ICA reaction as previously suggested [151], but the contribution of the ICA analysis is minor and does not seem cost effective at type 1 diabetes diagnosis but might be suggested as the last analysis in islet autoantibody negative patients with a suspected autoimmune type 1 diabetes. However, since multiple autoantibodies are important in assessing the risk for progression to type 1 diabetes [29, 70, 75-77, 81, 152], we believe that ICA is important in screening of patients for prevention studies [81].

Can the Medizym ICAscreen assay replace the ICA-IF assay?

The major drawback with the Medizym ICAscreen was that more positive samples were detected in this group of selected autoantibody negative children with diabetes compared with the ICA-IF assay. Consequently, since the sensitivity in
the Medizym ICAscreen was higher compared with the classical ICA-IF, the Medizym ICAscreen test requires that samples identified as positive are reanalyzed with ICA-IF to confirm ICA positivity.

Combinations of autoantibodies in multiple autoantibody positive non-diabetic children

After investigating associations between autoantibodies and different variables in children and adolescents at type 1 diabetes diagnosis, we wanted to further analyze autoantibodies in relation to descriptive parameters and metabolic control in non-diabetic children with multiple autoantibodies. Prior studies, primarily in children born in families with a first degree relative with type 1 diabetes, have shown that the number of islet autoantibodies is associated with an increased risk for impaired glucose metabolism [80, 135-136]. As the risk of progression to type 1 diabetes is high with multiple islet autoantibodies, non-diabetic children with GADA and at least one more islet autoantibody were invited to participate in the DiAPREV-IT study, the first prevention study with Alum-GAD (Diamyd®) with the aim to evaluate if the treatment may delay or prevent onset of type 1 diabetes. In paper IV we studied 47/50 pre-pubertal children from the DiAPREV-IT study at baseline (i.e. before any study drug had been given). We excluded three children from this baseline analysis since they were outliers in age. As GADA positivity was one of the inclusion criteria in this study, all patients had GADA with levels ranging between 35 and 50,000 U/ml (median 261 U/ml). The children were 4.0 – 9.2 (median age 5.1) years old and there were 25 (53%) boys.

At the first visit we found that the most common islet autoantibody after GADA was IA-2A, followed by ZnT8RA, ZnT8WA, IAA and ZnT8QA (as in Paper II). Double autoantibody-positivity was most common and three islet autoantibodies least common (Figure 11). As in paper I and II the presence of 2-6 islet autoantibodies was not affected by age.
Glucose metabolism was evaluated at baseline, before any treatment in the study had been given, with IvGTT and OGTT 8-65 days apart (median 21 days). Islet autoantibodies were measured at both occasions and we found that both the number and the levels of the autoantibodies changed between the two baseline samplings, despite the short time span between the visits. Two children became positive for IAA and ZnT8QA, respectively, between the visits. A total of 6 children lost one autoantibody - IAA was lost in two children, ZnT8RA in two children and ZnT8WA in one child. Interestingly, the child that gained ZnT8QA at the second visit lost IA-2A, resulting in a constant number of islet autoantibodies. Evaluating the level of the different autoantibodies, GADA was most stable as none of the children experienced a change in levels exceeding a doubling. There was a significant rise (≥2 times higher islet autoantibody level) in one or two islet autoantibody levels between the two visits in 8 children. The highest rise was a 10 fold increase in ZnT8QA, followed by a 9 fold increase in IA-2A level. Additionally, five patients had a significant fall (≥2 times lower islet autoantibody level) between the two visits in one islet autoantibody level, with the largest fall of 4 times in the ZnT8WA level. We believe that the fluctuation of islet autoantibodies between the two visits represents signs of an active on-going
autoimmune disease process. The ZnT8A were more common among these healthy young children than among the children with type 1 diabetes in paper I and II. Taking all three ZnT8A as one group, 35 (74%) were positive for the ZnT8A. ZnT8RA was found among 68%, ZnT8WA among 55%, and ZnT8QA among 47%, respectively (Figure 12).

![Figure 12. Frequency (%) of islet autoantibodies in the 47 healthy children, showing the distribution between normal and impaired glucose metabolism (Paper IV).](image)

A total of 22/34 (65%) of the ZnT8A positive children were triple ZnT8A positive as in Paper I and Paper II and we could again confirm a co-occurrence between all three ZnT8A (p-values ≤0.003). In our 47 children, ZnT8QA was not found to occur more often with IA-2A or IAA. Without using the ZnT8A, 6 children would only have been GADA positive and not able to participate in the secondary prevention study, since the inclusion criteria was GADA and at least one more islet autoantibody.
BMI, fasting plasma glucose, HbA1c and fasting C-peptide

All children had normal BMI, fasting plasma glucose, HbA1c and fasting C-peptide. The median BMI was 16.1 (range 13.7-19.3). Using the Swedish standardized curves for length, weight and BMI the standard deviation of BMI was plotted. Analyzing fasting C-peptide and BMI we found a tendency of lower C-peptide levels in the children with a BMI of less than -1 SD compared to those with a BMI above +1 SD, but the difference between the groups was not significant. There was also a trend of lower fasting C-peptide in the children younger than five years of age.

Glucose metabolism in non-diabetic children

None of the children developed diabetes during this study period. All children had a normal IvGTT-based k-value >1.2 and the insulin levels were normal both at the start and at the end of IvGTT. However, a total of 14 children (30%) had a low FPIR-value of <30 (median 43, range 8-145 µU/mL) (Figure 13). Fasting glucose was within normal limits in all children. In the OGTT we found a rise in p-glucose between 0 and 120 min in all but 5 children, with a median value at 120 min of 6.7 mmol/L. A total of 11/47 (23%) of the children fulfilled the criteria of impaired glucose tolerance (120 min p-glucose 7.8-11.0), and five of these children also had a low FPIR (Figure 13). The plasma C-peptide level and insulin level was normal both at the start and at the end of OGTT. The area under the curve (AUC) for C-peptide varied between 57.5 and 321.1, with significantly lower levels in the group with impaired glucose metabolism (p=0.009) (Table I in Paper IV).
Figure 13. Children with impaired glucose metabolism. A total of 5 children had impaired P-glucose in OGTT (≥7.8 but ≤11.1) and a low FPIR (≤30 pmol/L insulin). Another 9 children had low FPIR but normal 2h glucose in OGTT and 6 children had an impaired P-glucose in OGTT but a normal FPIR. None of the children had a k-value below 1.2.

Using standard definitions and normal values for fasting plasma glucose, fasting C-peptide, HbA1c, 2 hr OGTT plasma glucose together with k-value and FPIR in IvGTT we found that 20 (43%) children fulfilled at least one criterion for impaired glucose tolerance or reduced β cell function. We call this impaired glucose metabolism (Figure 13).
Does the type, level or number of autoantibodies predict the glucose metabolism?

We next added the question of whether we could find any differences between children with normal and impaired glucose metabolism in respect of the type or level of autoantibody or the number of autoantibodies with special focus on the three ZnT8A. We also wanted to investigate if the HLA genotypes could predict the glucose metabolism. Figure 13 demonstrates that 6/47 children had isolated impaired glucose tolerance on OGTT, 9/47 children had an isolated low FPIR, while five children had both these signs of impaired glucose metabolism. We believe that the 5/47 (11%) children with abnormal OGTT and IvGTT have a more advanced progression to disease, but future follow-up will be needed to confirm or reject this.

We could not confirm that the number of islet autoantibodies differed between children with normal and impaired glucose metabolism (Table II in Paper IV). However, both the frequency (Figure 12) and level of ZnT8QA (p=0.03 and 0.01, respectively), but not any of the other islet autoantibodies were increased in the impaired glucose metabolism group (Table II in Paper IV). We found no differences in the changes in islet autoantibody levels between the two visits between the children with normal and impaired glucose metabolism. The impaired glucose metabolism was not explained by age, gender or BMI (Table I in Paper IV).

The number and the levels of autoantibodies changed over a short period of time in this study. As many of the children are followed within the DiPiS and TEDDY studies, we know that some of the children have displayed other islet autoantibodies earlier in life. A weakness of this study is that all children have not been subjected to long term follow-up prior to the two visits. Therefore, it was not possible to include the number of previously lost islet autoantibodies in this study as this information was not available in all 47 children. This may be the reason for not being able to confirm that the number of autoantibodies was related to progressive β cell destruction.

With the exception of the HLA-DQ2/8 genotype that was found in 65% of the children with impaired glucose metabolism, compared to 33% (p=0.03) of the children with normal glucose metabolism, HLA did not explain the impaired glucose metabolism. The frequency of HLA-DQ2/8 was high in our study, explained by the fact that our subjects were mainly recruited from the population based studies DiPiS and TEDDY, where children with high risk HLA-DQ
genotypes are followed. Nevertheless, the finding that children with this genotype more often had impaired glucose metabolism is consistent with the previous finding that the HLA-DQ2/8 genotype is associated with an earlier age at clinical onset of type 1 diabetes [107].

Impaired glucose metabolism was more common in children with a first degree relative with type 1 diabetes 7/13 (54%) compared to those without heredity 13/34 (38%), however without significance. Unpublished observations in the TEDDY study indicate that children with first degree relatives have an increased risk for early clinical onset of type 1 diabetes compared to children from the general population.

Do we need to analyze all three ZnT8A?

ZnT8QA rarely occurs as a single islet autoantibody at the time of clinical onset of type 1 diabetes, and it has been suggested that it would be sufficient to measure ZnT8RA and ZnT8WA. In our studies we have shown that ZnT8QA can be found together with all the other islet autoantibodies and be the second autoantibody in otherwise single autoantibody positive children, and since multiple autoantibodies are used for prediction of type 1 diabetes, we believe that it is important to measure all three ZnT8A. Moreover, as the frequency of ZnT8QA (p=0.03), but not any of the other islet autoantibodies, was increased in the group with impaired glucose metabolism in this study, we conclude that it is important to analyze all three ZnT8A even in young children. The importance of ZnT8A measurements was also observed in the TrialNet study, where ZnT8A predicted diabetes independently of the other islet autoantibodies including ICA, age and HLA type [87].
9. Conclusion and Future Perspectives

The principal conclusions of the studies underlying this thesis (Papers I to IV) are that it is important to analyze all three ZnT8A at clinical onset of diabetes for classification of diabetes as well as in prediction of type 1 diabetes. As the analysis of all three ZnT8A can find 65% of type 1 diabetes patients at diagnosis and 2-3.4% of patients that without this analysis would have been considered as islet autoantibody negative, the ZnT8A analysis increases the diagnostic and predictive sensitivity of islet autoantibodies and the classification of diabetes. The ZnT8QA and the high risk HLA DQ2/8 are associated with impaired glucose metabolism in children with multiple autoantibodies and increased risk of developing type 1 diabetes. We suggest that both immunogenetic and individual islet autoimmunity markers, rather than number of islet autoantibodies, are important at inclusion in secondary prevention trials. We also believe that evaluation of baseline metabolic control is critical and should be taken into account before randomization in secondary prevention, otherwise the results of the studies might be affected by the fact that many children, already at inclusion, have impaired glucose metabolism and might not respond to the treatment. As children can both lose and gain autoantibodies during the pre-diabetic phase we think that it is important to take both the appearance and the loss of islet autoantibodies into account when the number of autoantibodies is used as a predictor of type 1 diabetes.

All variants of ZnT8A were positively associated with either DQ6.4 or DQ8 but dominantly negative with DQ2 in the BDD study, although we could only find a positive association between ZnT8RA and ZnT8WA and DQ6.4 in the Skåne study, probably because of the smaller number of patients. Analysis of ZnT8A in relation to the immunogenetic risk is important in order to uncover the mechanisms by which ZnT8 autoimmunity contributes to type 1 diabetes.

An isolated positivity for ICA despite negativity for GADA, IA-2A, IAA and ZnT8A suggests that still other yet unidentified autoantigen/autoantigens contribute to the ICA reaction and could explain autoimmunity in autoantibody negative patients. Most children diagnosed with diabetes in Sweden have autoimmune type 1 diabetes and 91% display at least one islet autoantibody.
Based on our data from the population-based and country-wide BDD study on 0-18-year-old diabetes patients we have implemented an algorithm for classification of childhood diabetes (Figure 16). In the first step GADA, IA-2A and HLA should be analyzed. Samples negative for GADA and IA-2A should next be analyzed for IAA and the three ZnT8A. With this model about 18% of Swedish children will reach the second step. Samples negative for GADA, IA-2A, IAA and ZnT8A, about 11% of the Swedish children, will thereafter be analyzed for monogenic diabetes (including MODY) unless this analysis has not already been done on clinical grounds. The responsible clinician will get a mail asking him or her to consider if the autoantibody negative patient might have type 2 diabetes, monogenic diabetes, including MODY, or secondary diabetes (Figure 16). The only autoantibody not included in this algorithm is ICA. In the Skåne study we found that 10/686 (1.5%) of the type 1 diabetes patients had ICA but no other islet autoantibody but in the BDD study we found only 10 patients (0.3%) that displayed ICA among the GADA, IA-2A, IAA and ZnT8A negative patients. A potential weakness of the algorithm for classification of diabetes is that some patients may be ICA positive but negative for all other autoantibodies, and it cannot be excluded that some of these patients may have autoantibodies to less frequent autoantigens such as VAMP2 and NPY [127].
Figure 16. Algorithm for classification of diabetes in the 0-18-year-olds.

As the incidence of type 1 diabetes is still rising worldwide and we all hope to learn more about the disease and the pathogenesis of the disease, large-scale studies in the general population should be undertaken. These should include analyses of all islet autoantibodies and HLA and in selected cases OGTT and IvGTT. With these analyses we might be able to predict with higher accuracy
which patients will develop type 1 diabetes and if these patients more often have the highest risk HLA DQ 2/8 or if the HLA might not be that important any more. It is also of interest to find out if the ZnT8A, especially ZnT8QA is a better predictor of progression to type 1 diabetes and to learn more about zinc metabolism and its effect in diabetes, including the formation of ZnT8A. It will be very interesting to follow the 47 children in the DiAPREV-IT study and to find out to what extent the children with impaired glucose metabolism at inclusion in the study progress more often or faster to type 1 diabetes and how their autoantibodies change both in number and titer.
Diabetes beskrevs av Egyptierna redan 1500 före Kristus, då man smakade på urinen och konstaterade att den var söt. Under antiken introducerades namnet diabetes som kommer från grekiskan och betyder ”siphon” som innebär att man kisar mycket (sockret binder vatten och urinmängderna ökar). Diabetes är en stor sjukdomsgrupp med påtaglig variation. Vid typ 1 diabetes kan kroppen inte längre tillverka tillräckligt med insulin. Vid typ 2 diabetes (som framförallt drabbar vuxna/äldre människor) fungerar insulinet dåligt men finns oftast i ökad mängd. Insulin är en stor molekyl och kan inte tas upp av tarmen. Därför måste patienter med typ 1 diabetes ta insulin i sprutform, medan patienter med typ 2 diabetes ofta kan klara sig med tablettar om påverkar det insulin de själva utsöndrar. Typ 1 diabetes är en av de vanligaste kroniska sjukdomarna hos barn och ökar i hela världen. För att överleva och undvika skador av sjukdomen måste man ta insulinprutor till varje måltid.


Kroppen har en fantastisk reservförmåga. Vi har två lungor, två njurar osv trots att vi överlever och klarar oss bra med endast en lunga eller en njure. Först när ca 10-20 % av insulinproduktionen återstår, insjuknar man i typ 1 diabetes med symptom som ökad törst, ökade urinmängder, trötthet, suddig syn och i värsta fall syraförgiftning. Detta är ett livshotande tillstånd med svåra buksmärtor, kräkningar och tung andning orsakad av ett lågt pH i kroppen. Pga insulinbristen kan kroppen inte använda sig av sockret som energi. Som kompensation bryter kroppen ner fett och denna fettnedbrytning sker vid insulinbrist och leder till ett sjunkande pH.


Dessa autoantikroppar och HLA gener går att mäta när som helst i livet. Genom att titta på antalet autoantikroppar, vilka autoantikroppar och hur höga nivåer av dessa autoantikroppar som varje patient har, kan man tillsammans med den genetiska HLA-profilen göra en riskbedömning. I vissa fall föreligger nästan 100 % risk att utveckla diabetes. En enstaka autoantikropp kan förekomma utan ökad risk att insjukna i diabetes. Med flera autoantikroppar är det vanligt att man insjuknar i typ
I diabetes inom några år, men enstaka individer kan ha många autoantikroppar i många år före diabetesdebut.

Syftet med denna avhandling var att titta på HLA och alla dessa autoantikroppar, framförallt ZnT8A, hos barn och ungdomar som precis insjuknat i typ 1 diabetes. Vi ville se på autoantikroppsforekomsten bland barn med diabetes och få en djupare insikt i om det finns skillnader utifrån ålder, kön och HLA. Vidare ville vi ta reda på om någon av autoantikroppen kunde anses ökade i många år före diabetesdebut.

Vi ville se på autoantikroppens förekomst bland barn med diabetes och få en djupare insikt i om det fanns skillnader utifrån ålder, kön och HLA. Vidare ville vi ta reda på om någon av autoantikroppen kunde anses ökade i många år före diabetesdebut. Slutligen ville vi undersöka friska barn med ökad risk att insjukna i diabetes för att ta reda på om det fanns några skillnader beroende på HLA och de olika autoantikropparna i barnens förmåga att bryta ner socker.

I det första arbetet undersökte vi 686 barn och ungdomar som insjuknat i typ 1 diabetes i Skåne mellan 1996 och 2005. Vi fann att ZnT8 autoantikroppar var viktiga och till hjälp för att bekräfta att dessa barn har typ 1 diabetes, då 26% av de barn som inte hade någon av de andra autoantikropparna hade ZnT8A. Vi fann också att dessa autoantikroppar var kopplade till en HLA-variant som tidigare inte har tolkats vara förknippad med typ 1 diabetes, men där vi bedömer att människor med just denna genetiska variant lättare kan bilda autoantikroppar mot ZnT8.


I det tredje arbetet ville vi titta på ICA – den autoantikroppsanalys som är dyrast och mest komplicerad att göra, men som ej ingick i den andra studien – för att avgöra om denna analys kunde bidra med ytterligare information hos de patienter

I det fjärde och sista arbetet undersökte vi 47 barn som följdes i en studie pga ökad risk att utveckla typ 1 diabetes utifrån HLA och autoantikroppar. På dessa barn gjorde vi olika undersökningar för att se om barnen redan hade tecken på att de hade svårt för att tolerera höga sockermängder som mått på en försämrad insulinproduktion och att de närmade sig en diabetesdebut. Vi fann att ZnT8QA var vanligare och förekom i högre nivåer hos de barn som hade sämst förmåga att hantera höga sockernivåer och att HLA DQ 2/8, det HLA som är förknippat med högst risk att insjukna i diabetes, är vanligare hos dessa barn.

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12. References


[26] Lan MS, Wasserfall C, Maclaren NK, Notkins AL (1996) IA-2, a transmembrane protein of the protein tyrosine phosphatase family, is a major autoantigen in insulin-dependent diabetes mellitus. Proc Natl Acad Sci U S A 93: 6367-6370


13. Appendix: Paper I-IV