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Autoantibodies as markers of beta-cell autoimmunity in children

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To my family,

with all my love

Abstract

Type 1 diabetes (T1D) is a chronic disease caused by destruction of the insulin producing beta-cells in the pancreas. The incidence of T1D has increased rapidly, especially in the Western world and among young children. The pathogenesis of T1D is not fully understood, but the beta-cells are believed to be destroyed by an autoimmune process initiated years before the onset of T1D. During this pre-clinical period, autoantibodies to insulin (IAA), glutamic acid decarboxylase (GADA) and the tyrosine phosphatase-like protein IA-2 (IA-2A) can be detected and are used to identify individuals at risk of T1D. The major genetic determinant for T1D is the HLA class II genes, but also polymorphism in the insulin gene and CTLA-4 gene are associated with T1D. The risk genes cannot explain the rapid increase in incidence of T1D, therefore a role for different environmental factors has been suggested.

The aim was to study the prevalence of beta-cell autoantibodies in children from the general population in relation to known genetic and environmental risk factors, and in young patients with T1D in high and low incidence areas.

Short duration of breast-feeding was associated with an increased risk of developing beta-cell autoantibodies in children from the general population at 5-6 years of age. We found an association between positivity for GADA and/or IAA at the age of 5-6 years and a short duration of total breast-feeding, and also between positivity for GADA, IA-2A and/or IAA and a short duration of exclusive breast-feeding. Our findings suggest that breast-feeding has a long term protective effect on the risk of beta-cell autoimmunity in children from the general population. The T1D related risk genes were not associated with beta-cell autoantibodies other than GADA in children from the general population at 5-6 years of age. Children with the DR4-DQ8 haplotype were more often positive for GADA than children without this haplotype. We found no association of GADA with DR3-DQ2 haplotype or between these two haplotypes and any of the other autoantibodies. Our results suggest that beta-cell autoimmunity in children from the general population is not strongly associated with any risk genes of T1D other than DR4-DQ8. In the non-diabetic children with allergic heredity GADA was detectable in almost all children, IA-2A in about half and IAA in 10% of the children. The levels low of these autoantibodies fluctuated with age and different patterns of fluctuations were seen for GADA and IA-2A, which may reflect differences in the immune response to the autoantigens. In patients with newly diagnosed T1D, we found some differences between patients from a high incidence country (Sweden) and a country with a lower incidence (Lithuania). Among the Swedish patients, the prevalence of IAA and GADA or multiple autoantibodies was higher than in Lithuanian patients. The risk genes DR4-DQ8 and the heterozygous high risk combination DR4-DQ8/DR3-DQ2 was more common among the Swedish patients than Lithuanian patients. Patients with low levels of IAA had higher levels of HbA1c and ketones, indicating that patients without IAA or with low levels of IAA have a more severe onset of T1D. Our findings indicate that beta-cell autoimmunity is more pronounced in a high incidence area compared to an area with a lower incidence.

In conclusion, short duration of breast-feeding is a risk factor for beta-cell autoantibodies in children from the general population, and the beta-cell autoantibodies in these children are not associated with specific risk genes. Children with newly diagnosed T1D in a high incidence area carry risk genes and have autoantibodies more often than newly diagnosed children from an area with a lower incidence, perhaps indicating different disease phenotypes.

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ABBREVIATIONS

ABIS	all babies in South-east Sweden
APC	antigen presenting cell
BB rat	BioBreeding rat
CD	celiac disease
CI	confidence interval
CTLA-4	cytotoxic T lymphocyte associated antigen 4
CV	coefficient of variation
CVB4	Coxsackie virus, serotype B4
DAISY	diabetes autoimmunity study in the very young
DASP	diabetes auto-antibody standardization program
DEBS	diabetes and environment around the Baltic sea
DIPP	type 1 diabetes prediction and prevention study
DPT-1	diabetes prevention trial type-1
ELISA	enzyme-linked immunosorbent assay
ENDIT	European diabetes intervention trial
GADA	autoantibodies to glutamic acid decarboxylase
HbA1c	haemoglobin A1c
HLA	human leukocyte antigen
IA-2A	autoantibodies to tyrosine phosphatase like protein IA-2
IAA	autoantibodies to insulin
ICA	islet cell antibodies
IDDM	insulin dependent diabetes mellitus
IFN	interferon
IL	interleukin
INS	insulin gene
kD	kilo Dalton
LADA	latent autoimmune diabetes in adults
MHC	major histocompatibility complex
MODY	maturity-onset diabetes of the young
NIDDM	non-insulin dependent diabetes mellitus
NOD mice	non-obese diabetic mice
RA u/ml	RA units/ml; the sample used for standard is denoted RA
SNP	single nucleotide polymorphism
T1D	type 1 diabetes
T2D	type 2 diabetes
TCR	T cell receptor
TEDDY	the environmental determinants in diabetes of the young
TRIGR	trial to reduce IDDM in the genetically at risk
WHO	World Health Organization
VNTR	variable number of tandem repeats

ORIGINAL PUBLICATIONS

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

- I. Hanna Holmberg, Outi Vaarala, Karin Fälth-Magnusson and Johnny Ludvigsson.
Induction of diabetes-related autoantibodies below cut-off for positivity in young non-diabetic children.
Ann N Y Acad Sci, 2003. 1005: p. 269-74

- II. Hanna Holmberg, Outi Vaarala, Vaiva Sadauskaitė – Kuehne, Jorma Ilonen Žilvinas Padaiga and Johnny Ludvigsson.
Higher prevalence of autoantibodies to insulin and GAD₆₅ in Swedish compared to Lithuanian children with type 1 diabetes.
Accepted for publication in Diabetes Res Clin Pract

- III. Hanna Holmberg, Jeanette Wahlberg, Outi Vaarala and Johnny Ludvigsson.
Short duration of breast feeding as a risk-factor for beta-cell autoantibodies in five year-old children from the general population.
Submitted to Brit J Nutr

- IV. Hanna Holmberg, Outi Vaarala, Jorma Ilonen and Johnny Ludvigsson.
HLA haplotypes and insulin gene VNTR polymorphism in relation to markers of beta-cell autoimmunity in children from the general population.
Manuscript

REVIEW OF THE LITERATURE

History of type 1 diabetes

Insulin is produced in the beta-cells in pancreatic islets of Langerhans. The insulin is needed for glucose to enter all cells and be used as a source of energy. An individual with diabetes suffers from insulin deficiency and hence an increased blood glucose. When blood glucose reaches a certain level, the kidneys are unable to retain the glucose and some is secreted with the urine. This causes symptoms like increased urine flow, increased thirst and weight loss. If not treated with insulin, the individual will die of starvation since the body cannot make use of the glucose.

The first known record of diabetes is from an Egyptian physician in 1552 BC who noted increased urine flow as the commonest symptom. An Indian physician used the sweet taste of the urine to diagnose diabetes in 400 BC. The name “diabetes” is a Greek word meaning to pass through and was first used around 250 BC. Later the Latin word “mellitus” meaning “honey” was added to describe the sweet urine. Two German physicians, Joseph Von Mering and Oskar Minkowski, discovered in 1889 that when they completely removed the pancreas of dogs, the dogs developed all the signs and symptoms of diabetes and died shortly afterward. In 1921, Frederick Banting and Charles Best repeated the work of Von Mering and Minkowski in the laboratory of John Macleod. They also showed that they could reverse the induced diabetes in dogs by giving them an extract from the pancreatic islets of Langerhans from healthy dogs. A biochemist, James Collip, improved the purification of the pancreatic extract. In 1923, Banting and Macleod received the Nobel Prize in Physiology or Medicine for the discovery and isolation of insulin. Banting later split his prize with Best and Macleod with Collip.

Definition and diagnosis of diabetes

According to the World Health Organization (WHO), diabetes mellitus is defined as “a metabolic disorder of multiple aetiology characterized by chronic hyperglycaemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action, or both” (WHO, 1999).

Diabetes can be classified into four major groups, according to WHO:

Type 1 diabetes (formerly insulin-dependent diabetes mellitus; IDDM) include the majority of cases due to pancreatic islet beta-cell destruction and are prone to ketoacidosis. Both incidences where the beta-cell destruction is caused by an autoimmune reaction or idiopathic cases are included. The process of beta-cell destruction ultimately leads to requirement of exogenous insulin to prevent death.

Type 1 diabetes (T1D) is usually characterised by markers of the autoimmune process, autoantibodies against glutamic acid decarboxylase (GAD_{65}), insulin, tyrosine phosphatase-like protein IA-2 (IA-2) or islet cell proteins. The progression of disease varies, often rapid in children and slower in adults. The slowly progressive form among adults is sometimes referred to as latent autoimmune diabetes in adults (LADA). The peak incidence occurs in childhood and adolescence, but it may occur at any age. There is a genetic predisposition and environmental factors are also important.

Type 2 diabetes (formerly non-insulin-dependent diabetes mellitus; NIDDM) is the most common form of diabetes and includes cases where diabetes is due to defects in insulin secretion and is often resistant to the action of insulin. The majority of the patients with type 2 diabetes (T2D) are obese, and the obesity causes or aggravates the insulin resistance. Exogenous insulin is usually not required for survival, at least initially. The hyperglycaemia is usually not severe enough to provoke symptoms, but increases the risk of long-term complications.

Gestational diabetes mellitus is defined as glucose intolerance first discovered during a pregnancy.

Other specific forms of diabetes include genetic defects of beta-cell function seen in maturity-onset diabetes of the young (MODY) and diseases of the exocrine pancreas.

The diagnosis of diabetes can be made from the symptoms among which increased urine flow, increased thirst and weight loss are included. In severe cases, the hyperglycaemia may cause ketoacidosis (WHO, 1999). When starving, the body breaks down its own protein and fat using it as an energy source. To provide energy for the brain, the liver makes ketone bodies (acetone and aceto-acetic acid), which are acidic. In addition to decreasing the pH of the blood, the osmolarity also changes, forcing water out of the cells to keep vital organs perfused and the result is dehydration. If not treated, this will lead to coma and death. The diagnosis of diabetes can not be made on hyperglycaemia alone, since this can be caused by for example, infections. Children usually present the severe symptoms mentioned above and the diagnosis of diabetes can be confirmed by a plasma glucose value of ≥ 12.2 mmol/l. In the case of less severe symptoms, a fasting venous plasma glucose value of ≥ 7.0 mmol/l or ≥ 6.1 mmol/l in capillary blood are considered as diagnostic cut off values for diabetes (WHO, 1999).

The long-term effect of diabetes include retinopathy, neuropathy and nephropathy as well as an increased risk of developing cardiovascular, peripheral vascular and cerebrovascular disease (WHO, 1999).

Epidemiology of type 1 diabetes

T1D is most common in Caucasians. In Europe, the incidence is higher in the northern countries compared to the southern countries, with Sardinia being an exception with a high incidence (Green *et al.*, 1992). World wide the incidence varies from 0.1 per 100 000 a year in China and Venezuela to 36.8 per 100 000 a year in Sardinia (Karvonen *et al.*, 2000). Finland has the highest incidence and in 1998 the annual incidence was 48.5/100 000 person-years (Podar *et al.*, 2001).

The overall increase in the annual incidence of T1D worldwide was 2.5% (Onkamo *et al.*, 1999), and an increase was seen both in countries with low and high incidence (Onkamo *et al.*, 1999; EURODIAB ACE study group, 2000). In a study of 0-34 year old Swedish patients with T1D during 1983-1998, no increase in incidence of T1D was found. Instead the median age when diagnosed had decreased and a shift towards earlier age at diagnosis could explain the increased incidence of T1D in childhood (Pundziute-Lyckå *et al.*, 2002). Also, in a study of the incidence of T1D in Europe, the relative increase in incidence was highest in children under 5 years of age (EURODIAB ACE study group, 2000).

Apart from the north-south gradient in the incidence of T1D in Europe, the incidence also varies between Western and Eastern Europe. The mean incidence in Sweden during 1978-1992 was 24.9 per 100 000 a year but in Lithuania it was 6.4 per 100 000 a year during 1983-1992 (Onkamo *et al.*, 1999). More recently, the incidence in Sweden was reported as almost 50 per 100 000 in 2002 in 5-14 year old children (The National Board of Health and Welfare Centre for Epidemiology, 2005), and as previously reported from the case-control study, Diabetes and Environment around the Baltic Sea (DEBS), the incidence in Lithuania varied between 8.0 and 10.2 per 100 000 a year during 1996-2000 (Sadauskaite-Kuehne *et al.*, 2004). The reason for the geographical differences in incidence within the European population is not known. Since the incidence of T1D is also increasing in different populations, T1D is less likely to be caused solely by genetic factors. Instead several different environmental factors, probably in combinations, may contribute to the increase in incidence and to the geographical differences.

Some aspects of the immune system

General aspects of the immune system: The immune system consists of a wide range of different white blood cells. These cells carry different receptors which enable them to perform different tasks in the immune response, all with the main purpose of eliminating pathogens and control inflammation. The signalling-molecules called cytokines are produced by activated cells and are of major importance in regulation of the immune responses. The immune response consists of two parts: 1) the innate or non-specific immune response involving granulocytes and macrophages which are

phagocytic and 2) the adaptive or specific immune response which involves the lymphocytes and providing the antigen-specific immunity that can follow the exposure to certain diseases or vaccinations.

The adaptive immune response consists of the lymphocytes (termed T cells and B cells). The lymphocytes interact with the different antigen presenting cells (APCs, for example dendritic cells) through their antigen-specific receptors. The lymphocytes become activated and proliferate; the T cells evolve into antigen-specific effector cells and the B cells into antibody-secreting cells.

T cells: The precursors of the T cells mature in the thymus. Here, the T cell receptors (TCRs) are generated and tested. Only lymphocytes that respond to antigen bound to the individual's own major histocompatibility complex (MHC) molecules will survive (positive selection). If the TCRs bind strongly to self-antigen, the lymphocytes will die (negative selection). In this way, tolerance to self-antigens is established. The T cells can be divided into different populations based on their surface molecules. T cells with the surface molecule cluster of differentiation 4 (CD4) are called TH1 or TH2 cells and if they carry CD8 they are called cytotoxic T cells. The T cells leave the thymus and circulate continually from the blood to the peripheral lymphoid tissues (lymph nodes, spleen and lymphatic tissues associated with mucosa). When APCs encounter antigens from for example a pathogen in the periphery, they carry the antigen to a lymph node and present it to an antigen-specific T cell, which will then proliferate and differentiate into an effector T cell.

The cytotoxic T cells recognize peptides from intracellular pathogens presented together with MHC I molecules, and kill the pathogen-infected cells. The TH1 and TH2 cells both recognize peptides derived from pathogens that are presented by APCs together with MHC II molecules. The TH1 cells are responsible for activating and attracting macrophages and cytotoxic cells to the site of infection and are thereby important in cell-mediated immunity against intracellular pathogens. The TH2 cells, on the other hand, are important in the humoral immune response since they induce antibody production by B cells to eliminate extracellular pathogens. The TH1 and TH2 cells produce different cytokines, TH1 cells produce mainly interferon- γ (IFN- γ) and TH2 cells produce interleukin-4 (IL-4), IL-5 and IL-9 (Romagnani, 2000).

The TH1 and TH2 cells have been shown to not only have different tasks in the immune system but also to inhibit the development of the opposing subset while promoting its own. Thereby IFN- γ inhibits TH2 cells (Gajewski *et al.*, 1988) and IL-4 inhibits TH1 cells (Hsieh *et al.*, 1992).

B-cells and antibodies: When activated by an antigen via its B cell receptor, the B cell differentiates into a plasma cell and produces antibodies, which are the secreted form of the B cell receptor. The main functions of the antibodies are to bind to and

neutralize pathogens or prepare them for uptake and destruction of phagocytes. The antibodies can be divided into five major classes or isotypes: immunoglobulin M (IgM), IgD, IgA, IgE and IgG. Here, I will focus on IgG antibodies. Each B cell expresses only one receptor specificity and hence only produces antibodies of one specificity, but the variety of receptors in one individual is almost endless. The IgG antibody is made out of two light- and two heavy-chains in a Y-shape, which consists of both constant and variable regions. The antigen-binding site of each antibody is made of the variable regions of both the heavy and the light chain. The two heavy chains are identical, as are the two light chains, giving the antibody two identical binding sites (Figure 1). The constant region with the C terminus is referred to as the Fc part of the antibody.

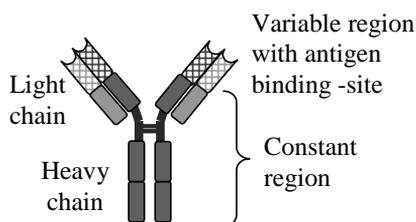


Figure 1: A schematic illustration of a IgG antibody, with two antigen-binding sites at the tip of the arms (modified from (Janeway *et al.*, 2005)).

The genes for the variable regions of the antibodies are encoded by sets of gene segments. During the development of the B cell in the bone marrow, one member of each set of gene segments are randomly joined to others by DNA recombination, which enables the individual to have a vast number of B cells with different antibody specificity.

The reader is referred to for example (Janeway *et al.*, 2005), where most of the abovementioned information can be found, for a more extensive survey of the immune system.

The immune system in young children: The immune system of the newborn infant is immature, although functioning in many aspects as reviewed in (Holt & Jones, 2000). For example, the production of antibodies is impaired at birth (Holt & Jones, 2000). Most of the IgG antibodies at birth are maternally acquired and the level decrease during the first 6-24 weeks of life (Fulginiti *et al.*, 1966). The T cell functions are also immature and mainly type 2 cytokines are produced in response to antigens. This TH2 polarisation is probably due to the down regulation of both foetal and maternal TH1 immune responses during the pregnancy (Holt & Jones, 2000).

Pathogenesis of type 1 diabetes

The pathogenesis of T1D is not fully understood, but is generally considered to be caused by an autoimmune destruction of the insulin producing beta-cells in the pancreas. The autoimmune process destroying the beta-cells may be initiated several years before diagnosis of T1D and cause a decrease in the beta-cell mass. Eventually the low production of insulin causes the clinical symptoms of T1D (Figure 2).

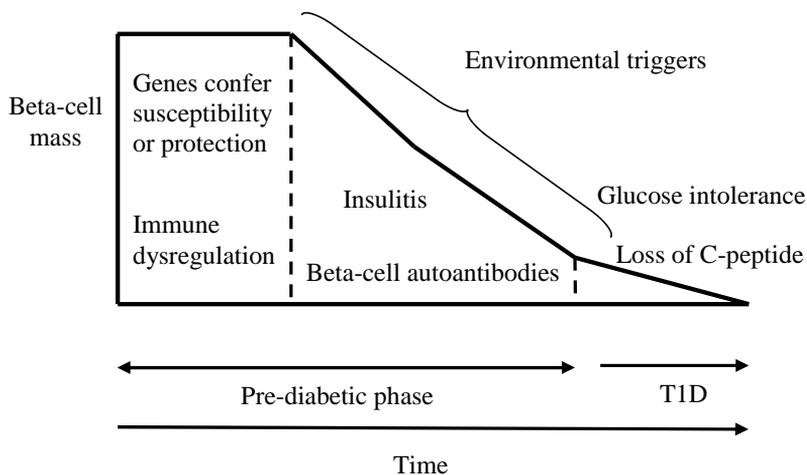


Figure 2: A model of the pathogenesis and natural history of T1D. The interaction of genes and immune system with different environmental factors may trigger a response against the beta-cells. As insulinitis develops, the beta-cell mass decreases and autoantibodies appear, as the individual progresses to T1D (modified from (Atkinson & Eisenbarth, 2001)).

Animal models of T1D, mainly the non-obese diabetic (NOD) mice and the BioBreeding (BB) rat, have given extensive information about the autoimmune process in the animal models. For example, T1D could be induced at an early age by transferring spleen T cells from diabetic NOD mice into neonatal NOD mice (Bendelac *et al.*, 1987). The autoimmune process causing an inflammation in the beta-cells (insulinitis) is characterised by infiltration of different inflammatory cells. In human insulinitis, the infiltrate consists of CD8⁺ cytotoxic T cells (Gepts, 1965; Imagawa *et al.*, 1999), CD4⁺ TH cells and macrophages (Moriwaki *et al.*, 1999). In Japanese patients with T1D, pancreatic biopsies have shown expression of Fas on the beta-cells and of Fas ligand on the infiltrating cytotoxic T cells. The interaction between Fas and Fas ligand may trigger selective apoptosis of the beta-cells (Moriwaki *et al.*, 1999). The autoreactive T cells probably play an important role in the pathogenesis of T1D, although several other cell types and immune mediators act together with these T cells in the autoimmune process.

During the pre-clinical period, the presences of autoantibodies directed against beta-cell antigens are markers of the autoimmune process. These autoantibodies can be detected years before diagnosis of T1D and may be used to identify individuals at risk of T1D.

Islet cell antibodies (ICA)

In 1974, autoantibodies of IgG class directed against pancreatic tissue were reported in patients with diabetes (Bottazzo *et al.*, 1974; MacCuish *et al.*, 1974). These antibodies became known as islet cell antibodies (ICA) and were widely used to study the pathogenesis of T1D and as a marker of beta-cell autoimmunity. The antibodies were detected by immunofluorescence, a labour intensive technique, which also proved difficult to standardize. ICA comprise a number of antigens, including GAD₆₅ and IA-2 but not insulin (Myers *et al.*, 1995; Leslie *et al.*, 1999).

ICA are detected in 71.0-86.0% of patients with newly diagnosed T1D (Verge, Howard, Rowley *et al.*, 1994; Sabbah *et al.*, 1999; Strebelow *et al.*, 1999; Borg *et al.*, 2002). The prevalence of ICA in individuals at increased risk of T1D (first degree family members or children with HLA risk genotypes) varies between 7.0-7.9% (Verge *et al.*, 1996; Kulmala *et al.*, 1998; Kukko *et al.*, 2005), while the prevalence in the general population (schoolchildren) has been reported 0.9% and 2.8% in two studies (Strebelow *et al.*, 1999; Kulmala *et al.*, 2001).

Autoantibodies to glutamic acid decarboxylase (GADA)

In 1982, autoantibodies were shown to immunoprecipitate a 64 kilo Dalton (kD) pancreatic protein in children with T1D (Baekkeskov *et al.*, 1982). Later, it was found before onset of T1D (Baekkeskov *et al.*, 1987) and was identified as the γ -amino butyric acid (GABA) synthesizing enzyme glutamic acid decarboxylase (Baekkeskov *et al.*, 1990). There are two isoforms of glutamic acid decarboxylase (GAD), GAD₆₅ and GAD₆₇ with the molecular weights of 65 and 67 kD (Bu *et al.*, 1992), although the GAD₆₅ isoform has been regarded as the primary autoantigen in humans (Hagopian *et al.*, 1993). Since the molecular cloning of GAD₆₅ (Karlsen *et al.*, 1991) several assays to detect autoantibodies against GAD₆₅ (GADA) have been developed, for example a radioligand binding assay with immunoprecipitation (Grubin *et al.*, 1994). It has been demonstrated that GADA most frequently binds to the central and C-terminal region of the GAD₆₅ molecule (Kaufman *et al.*, 1992; Richter *et al.*, 1993).

GADA are detected in 69.0-80.2% of patients with newly diagnosed T1D (Grubin *et al.*, 1994; Verge, Howard, Rowley *et al.*, 1994; Falorni *et al.*, 1995; Sabbah *et al.*, 1999; Strebelow *et al.*, 1999; Borg *et al.*, 2002). The prevalence of GADA in individuals at increased risk of T1D (first degree family members or children with HLA risk genotypes) varies between 3.2-10.7% (Verge *et al.*, 1996; Kulmala *et al.*,

1998; Barker *et al.*, 2004; Hummel *et al.*, 2004; Kukko *et al.*, 2005), while the prevalence in the general population (schoolchildren) varies between 0.5-3.0% (Strebelow *et al.*, 1999; Kulmala *et al.*, 2001; Marciulionyte *et al.*, 2001; LaGasse *et al.*, 2002).

As the enzyme of GABA, GADA is associated with the central and peripheral nervous system (Ellis & Atkinson, 1996).

Autoantibodies to tyrosine phosphatase-like protein IA-2 (IA-2A)

Further analysis of the 64 kD pancreatic protein showed that it contained another antigen besides GAD₆₅. Following tryptic proteolysis, a 40 kD or a 37 kD fragment was found to bind antibodies other than the 50 kD fragment that bound to GADA (Christie *et al.*, 1990). The 40 kD or a 37 kD fragments were found to be derived from the intracellular domain of tyrosine phosphatase-like proteins IA-2 and IA-2 β respectively (Payton *et al.*, 1995; Lu *et al.*, 1996). The autoantibodies against IA-2 (IA-2A) are directed to the intracellular part of the protein (Kawasaki *et al.*, 1997; Xie *et al.*, 1997; Zhang *et al.*, 1997) or to the juxtamembrane region (Naserke *et al.*, 1998; Hoppu *et al.*, 2004). As with GAD₆₅, IA-2 is also found in the tissues of the nervous system (Zhang *et al.*, 1997; Dromey *et al.*, 2004).

IA-2A are detected in 59.0-85.7% of patients with newly diagnosed T1D (Bonifacio *et al.*, 1998; Kawasaki *et al.*, 1998; Sabbah *et al.*, 1999; Strebelow *et al.*, 1999; Borg *et al.*, 2002). The prevalence of IA-2A in individuals at increased risk of T1D (first degree family members or children with HLA risk genotypes) varies between 2.0-5.3% (Verge *et al.*, 1996; Kulmala *et al.*, 1998; Barker *et al.*, 2004; Hummel *et al.*, 2004; Kukko *et al.*, 2005), while the prevalence in the general population (schoolchildren) varies between 0.2-2.4% (Strebelow *et al.*, 1999; Kulmala *et al.*, 2001; Marciulionyte *et al.*, 2001; LaGasse *et al.*, 2002).

Insulin as an autoantigen

The insulin molecule consists of two chains, A and B, linked by two disulfide bridges. The structure of the human insulin molecule is similar to that of porcine and bovine insulin (differs with one and three amino acids, respectively), as reviewed in (Potter & Wilkin, 2000). Insulin is synthesized as preproinsulin molecule and following the cleavage of NH₂ terminal sequence, proinsulin is formed (Hutton *et al.*, 2002). In the secretory granules of the beta-cell, proinsulin is then cleaved to form insulin and connecting C-peptide. These proteins are present in a 1:1 ratio (Hutton *et al.*, 2002). Proinsulin has been reported to be an autoantigen in T1D, but less specific in prediction of T1D (Williams *et al.*, 1999), although elevated proinsulin autoantibodies in adult T1D patients has been reported (Narendran *et al.*, 2003). Transcription of the insulin gene and production of low levels of insulin/proinsulin has been reported in

tissues other than the beta-cell, for example in the human thymus (Pugliese *et al.*, 1997; Vafiadis *et al.*, 1997). In the thymus, T cells that bind strongly to self-antigens are deleted through the process of negative selection. This deletion is dose-dependent and therefore the level of insulin expression can influence whether T cells reacting to insulin are deleted or not, as reviewed in (Anjos & Polychronakos, 2004).

Several lines of evidence suggest that insulin is the most important of the T1D associated antigens. Insulin is a beta-cell specific antigen, unlike GAD₆₅ and IA-2. In studies of the NOD mice, infiltrating T lymphocytes targeting the insulin B chain (Wegmann *et al.*, 1994) and islet-derived CD8⁺ T cells targeting the insulin B chain has been reported (Wong *et al.*, 1999). NOD mice with a modified insulin gene was recently reported to show no signs of immune response towards the beta-cells (Nakayama *et al.*, 2005). In humans, it has recently been reported that insulin A chain may be the trigger of the autoimmune response. In two studies, the lymphocytes from lymph nodes of HLA-DR4 positive T1D patients (Kent *et al.*, 2005) or the lymphocytes from a T1D patient (Mannering *et al.*, 2005) recognized the insulin A chain.

Autoantibodies to insulin (IAA)

Autoantibodies to insulin, by definition, develop in individuals before insulin treatment is commenced. It is generally considered that a sample taken within 14 days of the first insulin injection contains IAA since antibodies to the exogenous insulin has not yet developed (Bingley *et al.*, 2003; Falorni & Brozzetti, 2005). Autoantibodies to insulin in newly diagnosed T1D patients were first reported in 1983 (Palmer *et al.*, 1983). Later, it was reported that the levels of IAA correlate inversely with age (Arslanian *et al.*, 1985; Vardi *et al.*, 1988), and that IAA often is the first autoantibody to appear in children as a sign of beta-cell autoimmunity (Ziegler *et al.*, 1999), suggesting that insulin is an important autoantigen at least in young children.

IAA are detected in 43.0-70.0% of patients with newly diagnosed T1D (Verge, Howard, Rowley *et al.*, 1994; Williams *et al.*, 1997; Sabbah *et al.*, 1999; Strebelow *et al.*, 1999; Borg *et al.*, 2002; Williams *et al.*, 2003). The prevalence of IAA in individuals at increased risk of T1D (first degree family members or children with HLA risk genotypes) varies between 3.5-8.3% (Verge *et al.*, 1996; Kulmala *et al.*, 1998; Barker *et al.*, 2004; Hummel *et al.*, 2004; Kukko *et al.*, 2005), while the prevalence in the general population (schoolchildren) varies between 0.9-3.0% (Strebelow *et al.*, 1999; Kulmala *et al.*, 2001; Marciulionyte *et al.*, 2001; LaGasse *et al.*, 2002).

Analyses of beta-cell autoantibodies - methodological issues

Fluid phase radiobinding assays of GADA have generally demonstrated higher disease sensitivity compared to enzyme-linked immunosorbent assay (ELISA) (Schmidli *et al.*, 1995), which is seldom used (Bingley *et al.*, 2003). This may be due to the fact GAD₆₅ is adhered to the plastic in the ELISA and this may change the conformation of the epitopes (Schmidli *et al.*, 1995). Also in the case of IAA, the superiority of the radiobinding assay over ELISA has been reported (Levy-Marchal *et al.*, 1991; Greenbaum *et al.*, 1992). The fluid phase radiobinding assay is affinity dependent and favours the binding of high affinity IAA as opposed to ELISA which allows simultaneous binding of IAA of varying affinity (Wilkin, 1990). The radiobinding assay was reported to detect disease-associated IAA in the fourth international workshop (Greenbaum *et al.*, 1992).

Genetic risk of type 1 diabetes

Hereditary risk of T1D

First degree relatives of patients with T1D are at increased risk of developing diabetes compared to the general population. The risk of T1D in the general population is approximately 0.5%, the risk for offspring of a diabetic mother is 1-4%, the risk for offspring of diabetic fathers is 6-9% and the risk for siblings is approximately 6% as reviewed in (Hawa *et al.*, 2002; Redondo & Eisenbarth, 2002). However, the risk of T1D in relatives increase if the proband is diagnosed before 5 years of age (Gillespie *et al.*, 2002). Also, the concordance rate for monozygotic twins (meaning both twins have T1D) range from 21% to 70% and the concordance rate in dizygotic twins from 0% to 13% which is similar to singletons, as reviewed in (Redondo & Eisenbarth, 2002). Taken together, the data suggests a genetic component in the development of T1D.

The human leukocyte antigen complex (HLA)

The most important genes in defining risk of T1D are located within the human leukocyte antigen (HLA) complex on chromosome 6p21. The HLA genes provide up to 40-50% of the inheritable risk of T1D (Davies *et al.*, 1994; Noble *et al.*, 1996). The HLA complex is divided into three regions, class I, class II and class III. The HLA-DQ (Thorsby & Ronningen, 1993) and -DR loci in the class II region have a major influence on development of T1D (She, 1996). The DQ locus consists of two tightly linked genes; DQA1 and DQB1, encoding one alpha and one beta chain of the HLA molecule which together forms a heterodimer (She, 1996). If the HLA molecule is encoded by DQA1 and DQB1 genes of the same chromosome they are in *cis* position, but the genes can also be found on homologous chromosomes and are then found in *trans* position (She, 1996; Ilonen *et al.*, 2002). The alleles at one HLA locus are in

linkage disequilibrium, meaning they are non-randomly associated with alleles at other loci (She, 1996) and from a given DQB1 allele it is possible to deduce the DQA1 allele based on the linkage between the loci (Ilonen *et al.*, 2002).

Certain HLA class II alleles or haplotypes (combinations of alleles) show a strong association with development of T1D. Accordingly, the HLA haplotypes found more often among T1D patients than controls are considered predisposing, and when found more often among healthy individuals, they are then considered protective. The HLA-DQ heterodimers encoded by DQA1*0301-DQB1*0302 (DQ8) and DQA1*0501-DQB1*0201 (DQ2) have the strongest association with T1D in Caucasians (Kelly *et al.*, 2001). The DRB1*04 is in linkage disequilibrium with DQA1*0301 and DQB1*0302 and the DRB1*0301 is in linkage disequilibrium with DQA1*0501 and DQB1*0201 (Kelly *et al.*, 2001).

In Caucasians, the DQA1*0501-DQB1*0201-DRB1*0301, DQA1*0301-DQB1*0302-DRB1*0401, DQA1*0301-DQB1*0302-DRB1*0402, DQA1*0301-DQB1*0302-DRB1*0405 (Pugliese, 2004) and to some extent DQA1*0301-DQB1*0302-DRB1*0404 (Nejentsev *et al.*, 1998) are predisposing haplotypes (Table 1). The DQA1*0501-DQB1*0201-DRB1*0301 are commonly referred to as DR3-DQ2 and the DQA1*0301-DQB1*0302 haplotypes together with different DRB1*04 are referred to as DR4-DQ8. The highest risk for T1D are seen in individual heterozygous for DQA1*0501-DQB1*0201 and DQA1*0301-DQB1*0302 (DR3/DR4) (Ilonen *et al.*, 2002; Pugliese, 2004). In Caucasian individuals with T1D, 90-95% have been reported to carry DR3-DQ2 or DR4-DQ8 (Lernmark, 1994; Kelly *et al.*, 2001; Notkins & Lernmark, 2001) whereas 45-50% of the general population in USA carry these haplotypes (Lernmark, 1994; Notkins & Lernmark, 2001).

The haplotype DQA1*0102-DQB1*0602-DRB1*1501 confer a dominant protection, even in the presence of predisposing DQA1*0301-DQB1*0302 (Ilonen *et al.*, 2002) and it is observed in 20% of the general population (Todd, 1999) although it has been reported in about 1% of patients with T1D (Pugliese *et al.*, 1999; Todd, 1999) (Table 1).

The frequency of T1D predisposing HLA genotypes are not uniform within Caucasians, but varies in different populations in Europe (Rønningen *et al.*, 2001). When comparing frequencies of patients and healthy children from Finland, Greece and Hungary the frequencies of DQB1 genotypes were different, but in all three ethnic groups the heterozygous DQB1*02/*0302 genotype had a strong predisposing effect (Hermann *et al.*, 2004). On the other hand, comparing Finnish patients with patients from the Baltic region showed no significant difference in the frequency of T1D predisposing HLA alleles (Nejentsev *et al.*, 1998).

Table 1: Some of the HLA haplotypes in Caucasian populations and the association with risk of T1D.

Risk of T1D	HLA haplotypes
Highest risk	DQA1*0501-DQB1*0201 and DQA1*0301-DQB1*0302
Risk	DQA1*0501-DQB1*0201-DRB1*0301
Risk	DQA1*0301-DQB1*0302-DRB1*0401
Risk	DQA1*0301-DQB1*0302-DRB1*0402
Risk	DQA1*0301-DQB1*0302-DRB1*0405
Risk	DQA1*0301-DQB1*0302-DRB1*0404
Strong protection	DQA1*0102-DQB1*0602-DRB1*1501

The insulin gene (*INS*)

Another gene contributing to risk of T1D is the insulin gene (*INS*), located on chromosome 11p15.5. An association of the promoter region, a variable number of tandem repeats (VNTR), of insulin gene with T1D was found some 20 years ago (Owerbach & Nerup, 1982; Bell *et al.*, 1984). This region consists of polymorphic repeats of 14-15 base pairs (Bell *et al.*, 1981; Bell *et al.*, 1982), and the alleles of the *INS* VNTR are divided into three groups according to the number of repeats. In Caucasian populations there are mainly two groups; the class I alleles with 26-63 repeats and the class II alleles with 141-209 repeats (Bell *et al.*, 1984; Stead *et al.*, 2003). The alleles of intermediate length, class II, are rare in Caucasians (Bell *et al.*, 1984). A study of the polymorphisms around the *INS* VNTR reported the association within a 4.1-kb interval spanning *INS* with the VNTR and 10 single nucleotide polymorphisms (SNPs) in linkage disequilibrium with each other as well as with the *INS* VNTR (Lucassen *et al.*, 1993). The association of the shorter class I allele or associated SNPs with T1D has since been reported in several studies (van der Auwera *et al.*, 1995; Graham *et al.*, 2002; Perez De Nanclares *et al.*, 2003; Walter *et al.*, 2003).

Among the polymorphisms studied the -23 *HphI* (Undlien *et al.*, 1995) and the -2221 *MspI* were both found to be in concordance with the class I/III genotype of the VNTR (Lucassen *et al.*, 1993). The -23 *HphI* polymorphism is used as a genetic marker, were the A alleles are in strong linkage disequilibrium with the class I alleles of the *INS* VNTR and T alleles with class III alleles (Bennett *et al.*, 1995; Barratt *et al.*, 2004; Hermann *et al.*, 2005). The -2221 *MspI* is also used as a genetic marker, were the C alleles are in strong linkage disequilibrium with the class I alleles of the *INS* VNTR and T alleles with class III alleles (Barratt *et al.*, 2004). These SNPs are commonly used as surrogate markers for the *INS* VNTR polymorphism and we therefore tested the insulin VNTR by using -23 *HphI* and -2221 *MspI* in Papers II and IV.

The cytotoxic T lymphocyte associated antigen -4 gene (*CTLA-4*)

The cytotoxic T lymphocyte associated antigen-4 (*CTLA-4*) plays an important role in the activation of T cells. The activation of naïve antigen specific T cells by antigen presenting cells (APCs) occurs when the APC presents both a specific antigen to the T cell receptor (TCR) and a co-stimulatory molecule (B7.1/B7.2, also denoted CD80/CD86) to the CD28 molecule (Chambers, 2001; Egen *et al.*, 2002). This activation causes the T cell to expand and differentiate into effector cells (Chambers, 2001; Egen *et al.*, 2002). Shortly after the T cell is activated, *CTLA-4* is produced (Egen *et al.*, 2002; Saito & Yamasaki, 2003). *CTLA-4* suppresses the activation of the T cell both by having a 10-20 fold higher affinity for CD80/CD86 than CD28 and by inducing inhibitory signals (Egen *et al.*, 2002; Saito & Yamasaki, 2003).

An association of the *CTLA-4* gene and T1D was reported some 10 years ago (Nistico *et al.*, 1996). A point mutation at position 49, an A-G transition, in the *CTLA-4* gene was detected and the frequencies of the G alleles and G/G genotype were increased in Italian and Spanish patients with T1D compared to controls (Nistico *et al.*, 1996). Similar findings have been reported in patient/control comparisons in other Caucasian populations (Donner *et al.*, 1997; Marron *et al.*, 1997; Graham *et al.*, 2002) as well as in a Chinese population (Lee *et al.*, 2000). In populations at risk, siblings/offspring or children with HLA defined risk genes, no association of beta-cell autoantibodies and *CTLA-4* polymorphisms were found (Hermann *et al.*, 2005; Steck *et al.*, 2005).

Although the *CTLA-4* +49 A/G SNP, used in Paper II of this thesis, has been shown to be associated with T1D in a meta-analysis (Kavvoura & Ioannidis, 2005), others have reported that it is not the functional polymorphism for T1D (Turpeinen *et al.*, 2003; Ueda *et al.*, 2003). Rather, the +49 A/G SNP may be used together with the CT60 A/G SNP (Ueda *et al.*, 2003).

The immunological effects of the genetic risk factors

The highly polymorphic HLA class II genes as a consequence encode molecules that are highly diverse. The HLA class II molecule is mainly expressed on the surface of professional APCs, responsible for presenting exogenous antigen to T cells. The extracellular antigen is phagocytised by an APC and degraded to peptides within endosomes. A peptide is then assembled with a HLA molecule and transported to the cell surface where the complex is presented to a T cell. The HLA molecule consists of one alpha and one beta chain, both of which form the peptide-binding cleft (Klein & Sato, 2000) and the conformation of the HLA molecule is affected by different HLA genes, generating differences in the peptide binding clefts of the HLA molecule (She, 1996; Thorsby, 1997). HLA molecules associated with susceptibility to T1D share similar traits in their antigen-binding clefts which may result in differences in their ability to bind to diabetogenic antigens (Kelly *et al.*, 2003). Certain HLA molecules

may more efficiently bind and present antigens to the T cell and thereby affecting the T cell response and repertoire (Thorsby, 1997). Another explanation is that HLA molecules associated with protection from T1D form more stable complexes with self-antigens in the thymus, leading to a more efficient deletion of autoreactive T cells (negative selection) (Kelly *et al.*, 2003). The HLA genes therefore have a major effect on the immune response, not only in T1D.

Since either the *INS* VNTR or other polymorphisms change the coding sequence of *INS*, their effect must be due to effects on the transcription of the gene. Transcription of the *INS* gene and the production of low levels of insulin/proinsulin occurs mainly in the beta-cells, but also to some extent in the thymus ((Pugliese *et al.*, 1997; Vafiadis *et al.*, 1997). As reviewed in (Pugliese & Miceli, 2002), the expression of self-antigens like insulin in the thymus may be necessary for the development of self-tolerance. The level of transcription for class III alleles is higher than for class I alleles (Pugliese *et al.*, 1997). Increased levels of insulin may induce more efficiently a negative selection of autoreactive T cells specific for insulin or improve the selection of regulatory T cells, thereby influencing the induction of tolerance to insulin.

The *CTLA-4* +49 A/G SNP affects the function of CTLA-4, as healthy individuals with G/G genotype show a decreased up-regulation of CTLA-4 compared to A/A genotype individuals (Maurer *et al.*, 2002). The decreased up-regulation of CTLA-4 may affect the ability to down-regulate the T cell responses. As reviewed in (Pugliese, 2004), the polymorphism influences the splicing of *CTLA-4* mRNA and individuals with G/G genotype show decreased levels of soluble CTLA-4 (sCTLA-4). Due to the decreased level of sCTLA-4, the CD28 may bind CD80/CD86 on the APCs to a higher extent thereby reducing the inhibition of the immune response (Pugliese, 2004) and render a down-regulation of the immune response difficult.

Prediction of type 1 diabetes

It is estimated that only about 15% of the newly diagnosed T1D patients have a first degree relative with the disease (Redondo, Eisenbarth, 2002). To predict development of T1D in the remaining 85%, other strategies are required. Since no intervention is yet available, the main purpose of characterising individuals at increased risk of developing T1D is for various research projects; prevention trials or etiological studies. Since the risk of T1D is 0.4% or higher (She & Marron, 1998) in Caucasian populations, selection based on heredity, genetic risk and/or autoantibodies also increases the number of affected individuals in the study population.

In studies of first degree relatives, the risk of developing T1D increases markedly when multiple autoantibodies are detected (Bingley *et al.*, 1994; Verge *et al.*, 1996; Hummel *et al.*, 2004). The risk of T1D in different studies in first degree relatives in

relation to autoantibody positivity is given in Table 2, modified from (Franke *et al.*, 2005).

Table 2: *The risk of T1D in first degree relatives in relation to autoantibody positivity reported in different studies (modified from (Franke et al., 2005)).*

Autoantibody positivity	Cumulative risk of T1D (over 5 years)
IAA	24-59%
GADA	12-52%
IA-2A	26-81%
ICA	29-63%
GADA and IAA	68%
IAA and IA-2A	100%
GADA and IA-2A	86%
Any 2 autoantibodies	25-100%
Any 3 autoantibodies	55-100%

Screening for HLA associated risk genotypes has been used in several different populations. In Finnish populations, the risk associated DQB1*0302 and *02 together with the three most important protective alleles (DQB1*0602, *0603 and *0301) have been used to grade risk of T1D (Ilonen *et al.*, 1996; Nejentsev *et al.*, 1999). Using this kind of strategy in the Type I Diabetes Prediction and Prevention project (DIPP) for screening the general population, about 77% of the children who developed T1D was identified as children at increased genetic risk (Kupila *et al.*, 2001).

Prediction and prevention studies

In the All Babies in south-east Sweden (ABIS) study children from the general population are followed, but several other studies have followed children at increased risk of T1D with frequent sampling and autoantibody analysis. The long period of beta-cell destruction has been considered as a “window of opportunity” when intervention could be used to delay or even stop the destructive process. Prediction of T1D is possible in children based on multiple autoantibody positivity in populations at risk but so far no intervention or prevention has been successful.

BABYDIAB: The BABYDIAB study is a prospective German multicentre study. Offspring of parents with T1D were recruited and samples were taken at 9 months, 2, 5 and 8 years of age. At 9 months of age, 1.4% of the children were positive for IAA, 0.5% for GADA and 0.2% for IA-2A. The frequencies of all three autoantibodies increased with age and at 5 years of age, 4.4% were positive for IAA, 4.4% for GADA

and 3.0% for IA-2A. At 5 years of age, 3.5% of the children had multiple autoantibodies. Of the 24 (1.5%) children who developed T1D by 5 years of age all but one child had multiple autoantibodies (Hummel *et al.*, 2004).

DAISY: The Diabetes Autoimmunity Study in the Young (DAISY) is a prospective cohort study in Denver, Colorado, USA. Young first degree relatives of patients with T1D (offspring or siblings) and newborns with high risk or moderate risk HLA genes were recruited. Samples were taken at 9, 15 and 24 months of age and annually thereafter. In children who were autoantibody positive, samples were taken with 3-6 months interval. If a sample was reported as positive it was re-analysed for confirmation. Of the confirmed positive children, 3.5% were positive for IAA, 3.2% for GADA and 2.0% for IA-2A. All 24 (1.1%) children who developed T1D, were confirmed positive for at least one autoantibody and 23/24 were positive for more than one autoantibody (Barker *et al.*, 2004).

DIPP: The Type I Diabetes Prediction and Prevention project (DIPP) is a Finnish study involving three centres. Newborns with high risk or moderate risk HLA genes were recruited. Samples were taken at 3, 6, 12, 18 and 24 months of age and annually thereafter. ICA was used as the initial screening test, and if positive, IAA, GADA and IA-2A was analysed (Kupila *et al.*, 2001). Persistent autoantibodies were defined as at least two positive samples in a row. By 5 years of age, 8.3% had IAA at least once, 4.6% had GADA and 3.3% had IA-2A. All 13 (1.3%) children who developed T1D by 5 years of age had at least two autoantibodies before diagnosis (Kukko *et al.*, 2005).

TEDDY: The Environmental Determinants in Diabetes of the Young (TEDDY) study involves centres in North America, Germany, Finland and Sweden. Newborn babies with increased genetic risk of T1D or with a first degree relative will be recruited. The long-term goal of the TEDDY study is the identification of environmental agents, for example infectious agents and dietary factors which trigger T1D in genetically susceptible individuals or which protect against the disease. No results are yet available (Lernmark *et al.*, 2005).

ENDIT: The European Diabetes Intervention Trial (ENDIT) was a double blind placebo-controlled trial, involving centres in Europe and North America. First degree relatives with high levels of ICA were recruited and allocated to oral nicotinamide or placebo for 5 years. There was no difference in the development of diabetes between the treatment groups (Gale *et al.*, 2004). Among the participants under 20 years of age, 57% were positive for IAA, 70% for GADA and 55% for IA-2A (European Nicotinamide Diabetes Intervention Trial Group, 2003).

DPT-1: The Diabetes Prevention Trial Type 1 (DPT-1) has recruited relatives of patients with T1D throughout the USA and Canada. First degree relatives were 2.5-40 years old and second degree relatives were 2.5-20 years old. The prevalence of GADA

or IA-2A among ICA positive relatives was 22.6% and 4.3% respectively (Yu *et al.*, 2001).

The ICA-positive relatives were then divided into two groups, one receiving parenteral insulin and the other oral insulin, but essentially no difference in prevention of T1D was found (Diabetes Prevention Trial--Type 1 Diabetes Study Group, 2002).

TRIGR: The Trial to Reduce IDDM in the Genetically at Risk (TRIGR) study is a multinational, randomized controlled trial involving over 70 centres in Canada, the USA, Europe and Australia. Newborns with an affected family member and with increased genetic risk are recruited. The mothers are encouraged to exclusively breastfeed the child for the first 6 months of life. If a mother is unable to exclusively breastfeed until the baby is 8 months of age, her child will be randomly assigned to one of two groups: formula with hydrolysed cow milk based formula versus a standard cow milk-based formula. The study will compare the rates of development of T1D in infants given hydrolysed cow's milk based formula versus a standard cow's milk-based formula (TRIGR, 2002).

In genetically selected populations, almost all children who progress to T1D have a pre-diabetes period when autoantibodies are detectable. Intervention would therefore necessitate sampling with short intervals in order to find children with autoantibodies at an early stage. This would be very laborious if done on a population level even if pre-screening for genetic risk is performed. It may also be worth while to consider if intervention should be offered only to those at increased risk, i.e. with both genetic risk and autoantibody positivity. Maybe, by then, it is too late to stop or delay the autoimmune process?

Environmental factors

The rapid increase in the incidence of T1D can not be explained by heredity or presence of risk genes, but rather by environmental factors. Several different environmental factors are suggested to trigger the autoimmune response, as reviewed in (Åkerblom *et al.*, 2002).

Breast-feeding: Breast-feeding has been proposed to have a protective effect in a number of studies and high frequency of breast-feeding was reported to be associated with low incidence of T1D (Borch-Johnsen *et al.* 1984; Dahl-Jorgensen *et al.* 1991). Short duration of exclusive breastfeeding has been reported to increase the risk of T1D (Virtanen *et al.*, 1992; Virtanen *et al.*, 1993; Verge, Howard, Irwig *et al.*, 1994; Sadauskaite-Kuehne *et al.*, 2004) as well as short duration of total breast-feeding (Borch-Johnsen *et al.*, 1984; Virtanen *et al.*, 1992). Duration of breast-feeding shorter than 3 to 4 months (Gerstein, 1994) and breast milk substitutes before the age of 3 months (Norris & Scott, 1996) has been demonstrated to be associated with

development of T1D in two meta-analyses. Differences in participation rates of T1D patients and controls may bias the studies, and maternal recall of the breast-feeding duration in retrospective studies may differ between cases and controls (Norris & Scott, 1996). Thus, prospective studies are needed to investigate the possible association between beta-cell autoimmunity and infant feeding in general and breast-feeding in particular. In a Finnish study, short duration of exclusive breast-feeding was associated with an increased risk of beta-cell autoantibodies in genetically predisposed children (T Kimpimäki *et al.*, 2001). However, the duration of breast-feeding was not associated with the increased risk of beta-cell autoantibodies in children with a first degree relative with T1D in Germany and Australia (Couper *et al.*, 1999; Hummel *et al.*, 2000; Ziegler *et al.*, 2003) or with either a first degree relative or increased genetic risk in USA (Norris *et al.*, 2003).

Cow milk: The risk of T1D implied by short duration of breast-feeding may be interpreted as early weaning to formula, which most often contains cow milk proteins. Early exposure to cow milk has been reported to increase the risk of T1D (Virtanen *et al.*, 1992; Kostraba *et al.*, 1993; Virtanen *et al.*, 1993). In epidemiological studies, increased risk of T1D has been associated with an early introduction of cow milk formula in infancy, indicating that triggering of the gut immune system in early infancy may contribute to the later development of beta-cell autoimmunity (Vaarala, 1999). In a prospective study of children at increased risk, the prevalence of beta-cell autoantibodies was lower in children weaned to a casein hydrolysate formula compared to children weaned to a control formula (Åkerblom *et al.*, 2005). In other prospective studies, early introduction of cow milk containing supplements did not affect the risk of beta-cell autoantibodies (Norris & Scott, 1996; Norris *et al.*, 2003; Ziegler *et al.*, 2003). High consumption of cow milk during childhood has also been associated with increased risk of beta-cell autoantibodies in both in first degree relatives and children from the general population (Virtanen *et al.*, 1998; Wahlberg *et al.*, 2006).

Bovine insulin & the gut immune system: One of the theories to explain how the risk of T1D is mediated by cow milk is by immunisation to bovine insulin. Children who received a cow milk-based formula had higher levels of IgG antibodies against bovine insulin compared to children who received either a formula containing hydrolysed formula or who were exclusively breast-fed (Vaarala *et al.*, 1998; Paronen *et al.*, 2000). The gut immune system is important for the induction of oral tolerance towards for example the bovine insulin molecule. The gut mucosa is immature in infants and young children, allowing passage of antigenic proteins that might trigger an immune response, and if the response is detrimental, it will affect the immune system (Kolb & Pozzilli, 1999). The association between T1D and cow milk may be due to the fact cow milk proteins is the first major source of foreign proteins the infant is exposed to (Kolb & Pozzilli, 1999).

Weight gain & the accelerator hypothesis: Weaning to formula has been associated with weight gain, and increased weight gain in infancy may be a risk factor for T1D (Johansson *et al.*, 1994; Hyppönen *et al.*, 1999). The accelerator hypothesis argues that T1D and T2D are the same disease, only distinguishable by the rate of beta-cell loss and which accelerators are responsible. The first accelerator is constitutionally or intrinsic; a high rate of apoptosis of the beta cells. The second accelerator is acquired; insulin resistance as a result of weight gain and physical inactivity. The third accelerator is the beta-cell autoimmunity and it develops only in a small and genetically defined subgroup of patients (Wilkin, 2001).

Other dietary factors: Different dietary factors have been suggested to be associated with development of T1D and beta-cell autoimmunity. Some examples are gluten, D-vitamin and nitrate and nitrite which are all reviewed in (Åkerblom *et al.*, 2002).

Viral infections: Viral infections have been suggested to play a role in the pathogenesis of T1D. The incidence of T1D shows a seasonal variation, which follows that of enterovirus infection, (Gamble & Taylor, 1969). The strongest evidence has been reported for congenital Rubella infection (Menser *et al.*, 1978), as about 20% of children born with Rubella develop T1D. Enterovirus, among them Coxsackie virus B4 (CVB4) has been suggested although a recent review concluded that it is uncertain if there in fact is a casual association between Coxsackie virus infection and T1D (Green *et al.*, 2004). Children with T1D have been shown to mount an impaired immune response towards CVB4 in vitro, as compared to healthy children (Skarsvik *et al.*, 2006).

Mechanisms of allergy and atopy

Allergy is a hypersensitivity reaction which is initiated by immunological mechanisms and may be antibody- and/or cell-mediated. The immune response is directed against antigens (referred to as allergens) reacting with antibodies, mostly of IgE class but also of IgG class (Johansson *et al.*, 2001). According to the definitions of European Academy of Allergology and Clinical Immunology (EAACI), “atopy” is the tendency to produce IgE in response to low doses of allergens and persons referred to as “atopic” also have hereditary predisposition to produce IgE to allergens (Johansson *et al.*, 2001). In addition to the production of IgE antibodies to allergen, a person with atopy or allergy show TH2 type responses to the allergen, characterised with production of for example IL-4, IL-5 and IL-13, as reviewed in (Kay, 2001). These cytokines produced by TH2 cells induce the differentiation and activation of eosinophils, stimulate B cells to produce high amounts of antibodies, including IgE, and stimulate the growth of mast cells and basophils, all of these which play an important part in development of allergic symptoms (Romagnani, 2000). During infancy and early childhood, allergic reactions are most often directed against food

allergens, reactions against inhalant allergens usually do not appear until school age (Hattevig *et al.*, 1993).

Celiac disease

Aberrant regulation of oral tolerance has been suggested in T1D by reports of enhanced immune reactivity to cow milk proteins in the patients with T1D, as reviewed in (Vaarala, 1999). In celiac disease (CD), or gluten sensitive enteropathy, a chronic inflammation in the small intestine results in villus atrophy. The inflammation is triggered by wheat gluten and antibodies to the autoantigen tissue transglutaminase are often detected in serum (Sollid, 2000). In children with T1D, CD is more common compared to the healthy population. The prevalence of CD in children with T1D varies between 1-16% in different studies, as reviewed in (Holmes, 2002). A similar genetic background can partly explain this increased prevalence, since both diseases are associated with HLA haplotype DRB1*0301 (associated with DQ2) and DRB1*0401 (associated with DQ8) (Sollid, 2000). Two prospective studies of beta-cell autoimmunity in genetically predisposed children have reported early introduction of gluten as a risk factor for beta-cell autoimmunity (Norris *et al.*, 2003; Ziegler *et al.*, 2003), indicating the importance of the gut immune system in both T1D and CD.

HYPOTHESES & AIMS OF THE THESIS

The general aims of the thesis were to explore the prevalence of beta-cell autoantibodies in children from the general population in relation to genetic and environmental risk factors, and also to explore the prevalence of beta-cell autoantibodies in young patients with T1D in high and low incidence areas.

The specific aims and hypotheses were:

- I. To study the natural course of beta-cell autoantibodies at low levels in a group of non-diabetic children. We hypothesised that an autoimmune reaction to beta-cell antigens may be initiated in young children but with autoantibodies at low levels, usually not considered positive.
- II. To study the prevalence of beta-cell autoantibodies at diagnosis in young patients from two areas with different incidences of T1D and to relate the beta-cell autoimmunity to known genetic risk factors and to the severity at onset. We hypothesised that beta-cell autoimmunity and genetic risk factors may be more common among the Swedish patients, partly explaining the higher incidence of T1D in Sweden.
- III: To study the prevalence of beta-cell autoantibodies in 5-6 year old children from the general population in relation to the duration of breast-feeding. We hypothesised that a short duration of exclusive breast-feeding may be associated with beta-cell autoimmunity, even several years after completing breast-feeding.
- IV: To study the prevalence of beta-cell autoantibodies in 5-6 year old children from the general population in relation to known genetic risk factors of T1D. We hypothesised that HLA related risk of T1D may be associated with increased prevalence of beta-cell autoimmunity in the children. We also hypothesised that prevalence of IAA may be increased in the children with the high risk insulin gene polymorphism.

SUBJECTS & METHODS

Study populations

Paper I: Diabetes-related autoantibodies in non-diabetic children

All children studied were born between February 1983 and July 1984 and had a family history of atopy; i.e. there was a personal history suggesting atopic disease in at least one of the family members. The 212 families were recruited from 10 maternity clinics to a prospective study to investigate the effect of maternal diet elimination during pregnancy on the development of atopic diseases in children (Fälth-Magnusson & Kjellman, 1987). Half of the mothers were randomized into a diet-group and avoided cow milk and egg during the third trimester, the others had a normal diet. All children were exclusively breast-fed until 3 months of age, or they received supplementary casein hydrolysate formula (Nutramigen®). Venous blood samples were drawn at 6 weeks, 6 months, 18 months and 5 years of age. The sera were stored at -20°C for later analysis. At least one blood sample was available from 205 children.

Parents filled in questionnaires at the follow-up at 6 weeks, 6 months and 18 months. They answered questions about duration of total breast-feeding, introduction of formula and infections. At the 18 months follow up all babies who had shown any sign of atopic disease or allergy were examined by a senior paediatric allergist. Their atopic disease until the age of 18 months was scored as “definite atopic” (26.9%) “probably atopic” (20.8%), “possibly atopic” (9.6%) and “not atopic” (42.6%) (Fälth-Magnusson & Kjellman, 1987).

Paper II: Swedish and Lithuanian children with type 1 diabetes

This study is part of the case-control study, Diabetes and Environment around the Baltic Sea (DEBS), conducted in south-east of Sweden, including the Skåne region and in the whole of Lithuania. All children, 0-15 years old, diagnosed with T1D during 01 08 1995 – 01 08 2000 in south-east Sweden and the Skåne region and during 01 08 1996 – 01 08 2000 in Lithuania were invited to participate in the study. Most of the children (83%) in south-east Sweden, half of the children (50%) in the Skåne region and all children in Lithuania diagnosed during the time period participated (Sadauskaite-Kuehne *et al.*, 2002). T1D was defined according to the WHO criteria (WHO, 1999). Patients from the original study population were included in this study based on the availability of samples from patients and from at least one family member (mother, father, siblings). From Sweden 96 patients were included, and from Lithuania 96 patients were included, matched for age and gender, since levels of IAA decrease with age (Vardi *et al.*, 1988) and the prevalence of GADA is higher among females (Verge, Howard, Rowley *et al.*, 1994). All the patients were 1-15 years old, median

age 9.0 years. The female/male ratio among the patients in the study sample was 51/49.

Serum samples were taken within 7 days of diagnosis and stored at -20°C until analysis of autoantibodies. Whole blood with EDTA was also stored at -20°C until genetic analysis was performed.

All children and their parents were asked to answer questionnaires at the time of diagnosis (Sadauskaite-Kuehne *et al.*, 2002; Sadauskaite-Kuehne *et al.*, 2004). Questions were asked about diabetes symptoms before diagnosis, infections, vaccinations, neonatal period including breast-feeding, autoimmune disease in the family and social factors (living conditions, parent's age, education and employment).

Paper III: Breast-feeding & autoantibodies in children from the general population

The current study was part of a prospective population-based follow-up study of all infants born during 01 10 1997 – 01 10 1999 in south-east Sweden (the ABIS study; All Babies in south-east Sweden) (Ludvigsson *et al.* 2001). Data on total and exclusive breast-feeding, dietary factors, hereditary factors, delivery, infections and mothers' age and education were obtained through questionnaires filled in at birth, and at 1, 2-3 and 5-6 years of age. Duration of total breast-feeding was defined as the period when any breast milk was given regardless of other food supplements and exclusive breast-feeding as the period when only breast milk was given. Duration of total breast-feeding was categorized into 0-3 months and 4 month or longer, and duration of exclusive breast-feeding into 1-3 months and 4 months or longer.

Autoantibodies were analysed in sera taken at 5-6 years of age and stored at -20°C. A sample for autoantibody analysis was available from 3788 children, 12 of them (0.3%) had developed T1D at the age of 5-6 years and were therefore excluded from the analysis. Data from questionnaires were available from 16 070 families at birth, 11 091 at 1 year, 8805 at 2-3 years and 7443 at 5-6 years follow-up.

Paper IV: Risk genes & autoantibodies in children from the general population

The current study was also part of the prospective ABIS study, see Paper III.

Autoantibody data at 5-6 years of age was available from 3776 children who had not developed T1D. Data on HLA haplotype were available from 714 and data on *INS*-23 polymorphism from 651 of these children. The genetic markers were analysed in whole blood samples taken at any of the three follow-up visits and stored at -20°C until analysed.

Methods

Paper I: Diabetes-related autoantibodies in non-diabetic children

Antigen preparation: The cDNA for GAD₆₅ was cloned into the pcDNA2 vector and cDNA for IA-2ic was cloned into pSP64 poly (A) vector (both gifts from Prof. Å Lernmark, Seattle). The cDNA was extracted from plasmid-carrying *E. Coli* using the Qiagen Plasmid Purification kit (KEBO labs, Stockholm, Sweden) and translated into ³⁵S-labelled protein using the TNT® SP6 Coupled Reticulolysate System (Promega, Madison, WI, USA). The methionine in the substrate was replaced with ³⁵S-methionine (Amersham Biosciences, Buckinghamshire, Great Britain). The ³⁵S-incorporation in the product was estimated by precipitation with trichloroacetic acid.

GADA and IA-2A assay: Antigen was diluted in a wash buffer (0.15M NaCl, 0.02M TRIS-HCl, pH 7.4) supplemented with BSA (0.1%) and Tween-20 (0.15%) to an activity of 20 000 cpm/50 µl. Serum samples were incubated with the antigen in a 1:25 dilution in duplicates, over night on a shaker at +4°C. This incubation was performed in a 96 round-bottom well plates (Labassco, Stockholm, Sweden). On each plate a standard (denoted RA) consisting of sample from one individual positive for both GADA and IA-2A diluted in wash buffer and comparable to the WHO standard were included, a well as a background sample and one positive and one negative control. Both standard and controls were serum samples from adults.

The following day, the IgG antibodies were precipitated with 15 µl Protein A-Sepharose (Zymed, San Francisco, CA, USA) diluted in 50 µl wash buffer in a 96 well filter plate (Millipore, Stockholm, Sweden) blocked with 1% BSA in TRIS-HCl buffer (0.15M NaCl, 0.02M TRIS-HCl, pH 7.4). After incubation for 45 min at +4°C, wells were washed with wash buffer using a vacuum manifold (Millipore, Stockholm, Sweden). After drying, scintillation fluid was added and the plates counted in a Micro-Beta counter (1450 Micro-Beta Tri-Lux counter, Wallac Inc., Turku, Finland). For all samples, mean and %CV were calculated. The results were expressed as RA units/ml (RA u/ml; the sample used for the standard is denoted RA) in relation to the standard curve. The lowest standard for GADA was 7.8 RA u/ml and for IA-2A the lowest was 5.5 RA u/ml. All samples with GADA below 7.8 RA u/ml or IA-2A below 5.5 RA u/ml were regarded as undetectable and were given the arbitrary value 3.9 RA u/ml and 2.8 RA u/ml respectively. Sera containing high levels of GADA or IA-2A were diluted to fall within the range of the standard curves. In inhibition assays, the binding between the radioactive antigens (GAD₆₅ or IA-2) and the autoantibodies was inhibited by addition of homologous antigen (unlabelled GAD₆₅ or IA-2).

The cut off limit for positivity at the 98th percentile of 1 year old non-diabetic Swedish children was 101.5 RA u/ml, corresponding to 35.4 WHO units for GADA and 36 RA u/ml, corresponding to 31.1 WHO units for IA-2A (N=4400). In DASP (Diabetes

Auto-antibody Standardization Program) for the year 2002, at the 98th percentile the specificity was 96% for GADA and 100% for IA-2A while the sensitivity was 82% for GADA and 54% for IA-2A. Inter-assay variation for negative and positive controls was 14% and 13% for GADA and 16% and 14% for IA-2A, respectively. I have in Paper I mistakenly given the arbitrary laboratory RA values instead of the WHO values as cut off for positivity. The correct RA values and WHO values are stated above.

IAA assay: Autoantibodies to insulin were determined by radiobinding assays according to (Williams *et al.*, 1997) with some modifications. Human insulin labelled with ¹²⁵I ((3-(¹²⁵I)iodotyrosyl)^{A14} insulin, from Amersham Biosciences, Buckinghamshire, Great Britain) was diluted in a TBT buffer (50mM TRIS-HCl, 1% Tween-20, pH 8.0) to an activity of 15 000 cpm/25 µl. Serum samples (7.5 µl) and labelled insulin were incubated in a 96 deep-well plate with shaking for 72 h at +4°C. On each plate a standard consisting of a pool of sera from eight newly diagnosed children (age 2-5 years, samples taken within 7 days of diagnosis) diluted in TBT buffer were included, a well as a background sample and one positive and two negative controls. The positive control and the two negative controls were serum samples from adults.

After 72 h, the IgG antibodies were precipitated with 15 µl Protein A-Sepharose (Zymed, San Francisco, CA, USA) diluted in 50 µl TBT buffer. The samples were washed five times by centrifugation at +4°C and aspirated using a vacuum manifold. Following transfer to 96 well isoplates, the samples were counted as above. For all samples, mean and %CV were calculated. The results were expressed as units/ml (u/ml) in relation to the standard curve. The detection of limit of the method was the lowest standard corresponding to 2 u/ml. All samples with IAA below 2 u/ml were given the arbitrary value 0.1 u/ml. All samples with IAA above 2 u/ml were tested in inhibition assay. Insulin labelled with ¹²⁵I insulin was added to quadruplicate samples. Equal volumes of unlabelled homologous insulin in excess were added to duplicate samples and the plates were incubated at 4°C with shaking for 72 h and then precipitated, washed and counted as above. Background counts were subtracted and specific counts were calculated by subtraction of counts of excess unlabelled insulin from counts of labelled insulin. The results were expressed as u/ml of IAA calculated in relation to a standard curve. Sera containing high levels of IAA were diluted to fall within the range of the standard curve.

The cut off limit for positivity corresponding to the 97th percentile of 72 non-diabetic Swedish schoolchildren (8-16 years old, median age 11) was 6 u/ml. In DASP in 2002, using 6 u/ml as the cut off level, the specificity was 98% and the sensitivity was 24%. Inter-assay variation for negative and positive controls was 13% and 9% in the screening assay and 15% and 14% in the inhibition assay.

General comment to autoantibody analyses: the protein A in Protein A-Sepharose binds to the Fc part of three of the four different human IgG subclasses: IgG₁, IgG₂ and IgG₄ antibodies (GE Health Care, formerly Amersham Biosciences; Hand book of Antibody purification).

Paper II: Swedish and Lithuanian children with type 1 diabetes

Antigen preparation: This assay was performed in the same way as in Paper I.

GADA and IA-2A assay: This assay was performed in the same way as in Paper I, with one exception. The antibodies were precipitated with 20 µl Protein A-Sepharose (nProtein A Sepharose 4 Fast Flow, Amersham Biosciences, Uppsala, Sweden).

The cut off for positivity at the 98th percentile of 4258 non-diabetic Swedish children (median age 3 years) was 105.1 RA u/ml, corresponding to 36.7 WHO units for GADA and 29.8 RA u/ml, corresponding to 28.5 WHO units for IA-2A. Sera containing high levels of GADA or IA-2A were diluted to fall within the range of the standard curves. In DASP in 2003, using these cut off values the specificity for GADA was 98% and 100% for IA-2A. The sensitivity was 78% for GADA and 48% for IA-2A. Inter-assay variation for negative and positive controls was 8% and 14% for GADA and 13% and 11% for IA-2A, respectively.

IAA assay: This assay was performed in a similar way as in Paper I, but all samples were analysed with an inhibition assay and samples were counted in a gamma counter.

Serum (7.5 µl) were analysed in quadruplicates and tested with a competition assay in deep-well plates. Human insulin labelled with ¹²⁵I was diluted in TBT-buffer to an activity of 25 000 cpm/25 µl and added to the quadruplicate samples. The same standard and negative controls as described above were used. The positive control consisted of a pool of sera from three newly diagnosed children (samples taken within 7 days of diagnosis). Equal volumes of unlabelled homologous insulin in excess or TBT-buffer were added to duplicate samples and the plates were incubated at 4°C with shaking for 72 h. The sample-antigen mixture was precipitated and washed as above. The samples were then transferred to vials and counted in a gamma counter (1282 Compugamma Wallac Inc., Turku, Finland). Calculations were performed as in Paper I.

The cut off for positivity at the 98th percentile of 114 non-diabetic Swedish children, (median age 10 years) was 6.45 u/ml. When using this cut off, we had specificity for IAA of 100%, while the sensitivity was 24% in DASP in 2003. Inter-assay variation was 5% for negative controls and 9% for positive controls.

Genetic analyses: The HLA genotyping as well as analyses of insulin gene and CTLA-4 gene polymorphisms were performed by the research group of Professor Jorma Ilonen in Turku, Finland.

HLA genotyping: From whole blood or from whole blood spots on filter paper, the HLA DQB1 typing was done by amplifying the second exon of the DQB1 gene by the polymerase chain reaction (PCR) using a primer pair with biotinylated 3' primer. The amplification product was bound to streptavidin-coated microtitre plates and denaturated by NaOH. After washing away the non-biotinylated DNA strand, the bound DNA was analysed using 12 different DQB1 probes in mixes of three probes labelled with various lanthanide (Europium, Samarium and Terbium) chelates. Specific signals for each label were measured by time-resolved fluorometry after washes and addition of enhancement solution. The different emission wave lengths and delay times were used for specific detection of each lanthanide label (Sjöroos *et al.*, 1995; Laaksonen *et al.*, 2002).

The DQA1 typing was done similarly in samples were it were deduced to be informative (in DQB1*02, *0301, *0303 positive samples). A mix of DQA1*0201, *03 and *05 probes labelled with various lanthanide labels were used as described previously (Sjöroos *et al.*, 1998; Laaksonen *et al.*, 2002).

The DR4 subtyping was done similarly from DQB1*0302 positive samples. Two mixes of three sequence specific probes were able to define DRB1*0401, *0402, *0403, *0404, *0405, *0408 and *0409 (Nejentsev *et al.*, 1999).

Individuals carrying DQB1*0302 and either DRB1*0401, *0402, *0404 or *0405 were combined and analysed together as DR4-DQ8 (non-DRB1*0403). Individuals carrying DQB1*02-DQA1*05 were analysed as DR3-DQ2. Also the high risk combination of DR4-DQ8 with DR3-DQ2 was used.

Insulin gene and CTLA-4 gene polymorphisms: The polymorphism in the insulin and CTLA-4 gene was detected as -2221 *MspI* C/T and CTLA-4 +49 restriction site polymorphisms. DNA from filter blood spots was amplified using primer pairs for insulin gene and CTLA-4 gene. The amplified product was transferred to streptavidin-coated microtitre plates as above and hybridised to two different lanthanide labelled probes, one for insulin gene and one for CTLA-4. Detection was done with time-resolved fluorometry as above (Haller *et al.*, 2004).

Polymorphism in the insulin gene was also detected as -23 *HphI* A/T restriction site polymorphism, which was analysed in a similar way.

HbA1c, ketones, and blood glucose: Data on haemoglobin A1c (HbA1c), ketones, and blood glucose were collected from the hospital records (Sadauskaite-Kuehne *et al.*, 2002). In Sweden HbA1c was measured by the DCA-2000 analyser (Bayer Diagnostics, Laverkevsen, Germany) and adjusted to a national standard which gives values about 1% lower than the DCTT standard (Kullberg *et al.*, 1996). In Lithuania, HbA1c was measured by the DCA-2000 without adjustments. In our calculations, the HbA1c values of the Swedish patients were therefore increased by 1%. Ketonuria was assessed with Ketostix reagent strips before the first insulin injection. Blood glucose was measured by standard glucose peroxidase reaction and the highest blood glucose value during the last 24 h before the first insulin injection was used in the statistical analysis.

Paper III: Breast-feeding & autoantibodies in children from the general population

Antigen preparation: This assay was performed in the same way as in Paper I and II.

GADA and IA-2A assay: This assay was performed in the same way as in Paper II.

The cut off for positivity at the 99th percentile of 5-6 years old non-diabetic Swedish children was 160.9 RA u/ml, corresponding to 61.4 WHO units for GADA (N=3251) and 5.6 RA u/ml, corresponding to 4.0 WHO units for IA-2A (N=3459). The cut off for positivity at the 95th percentile for GADA was 62.3 RA u/ml, corresponding to 20.9 WHO units. The cut-off for positivity was determined as the 99th percentile for IA-2A since the detection level of the IA-2A assay is 5.5 RA u/ml. In DASP for the year of 2005, at the 99th percentile the specificity was 99% for GADA and 100% for IA-2A while the sensitivity was 74% for GADA and 72% for IA-2A. At the 95th percentile, the specificity for GADA was 94% and the sensitivity was 80%. Inter-assay variation for negative and positive controls was 10% and 8% for GADA and 11% and 12% for IA-2A.

IAA assay: This assay was performed in the same way as in Paper II.

The cut off for positivity at the 99th percentile of 2201 non-diabetic Swedish children, (age 5-6 years) was 6.3 u/ml, and the cut off for positivity at the 95th percentile was 2.6 u/ml. In DASP for the year of 2005, at the 99th percentile the specificity for IAA was 100%, while the sensitivity was 24%. The corresponding figures at the 95th percentile was 97% and 34% respectively. Inter-assay variation for negative and positive controls were 11% and 8% respectively.

IAA above the 95th percentile or IA-2A above the 99th percentile was used as an outcome variable and marker of beta-cell autoimmunity. Also, GADA and/or IAA

above the 95th percentile or any one of GADA, IAA or IA-2A above the 99th percentile were used as outcome variables.

Paper IV: Risk genes & autoantibodies in children from the general population

GADA and IA-2A assay: This assay was performed in the same way as in Paper III.

The cut off for positivity at the 99th percentile of 5-6 years old non-diabetic Swedish children was 160.9 RA u/ml, corresponding to 61.4 WHO units for GADA (N=3251) and 5.6 RA u/ml, corresponding to 4.0 WHO units for IA-2A (N=3459). The cut off for positivity at the 98th percentile was 88.0 RA u/ml, corresponding to 30.8 WHO units for GADA. The cut-off for positivity was determined as the 99th percentile for IA-2A since the detection level of the IA-2A assay is 5.5 RA u/ml. In DASP in 2005, the specificity for GADA at the 99th percentile was 99% and the sensitivity was 74%. At the 98th percentile the specificity was 94% and the sensitivity was 76%. For IA-2A at the 99th percentile, the specificity was 100% and the sensitivity was 72%. Inter-assay variation for negative and positive controls were 10% and 8% for GADA and 11% and 12% for IA-2A.

IAA assay: This assay was performed in the same way as in Paper III.

The cut off for positivity at the 99th percentile of 2201 non-diabetic Swedish children, (age 5-6 years) was 6.3 u/ml, and the cut off for positivity at the 98th percentile was 4.2 u/ml. In DASP in 2005, we had specificity for IAA of 100%, while the sensitivity was 24% according to the 99th percentile and corresponding figures at the 98th percentile was 100% and 30% respectively. Inter-assay variation for negative and positive controls was 11% and 8%.

Single autoantibodies were used as markers for beta-cell autoimmunity. For this, GADA, IAA and IA-2A above 99th percentile and GADA and IAA above the 98th percentile were used as out-come variables. Also, the combinations of GADA and/or IAA at the 98th percentile and GADA, IAA and/or IA-2A at the 99th percentile were used as out-come variables.

Genetic analyses: The HLA genotyping as well as analysis of insulin gene polymorphism were performed by the research group of Professor Jorma Ilonen in Turku, Finland.

HLA genotyping: This assay was performed in the same way as in Paper II.

Individuals carrying DQB1*0302 and either DRB1*0401, *0402, *0404 or *0405 were combined and analysed together as DR4-DQ8 (non-DRB1*0403). Individuals carrying DQB1*02-DQA1*05 were analysed as DR3-DQ2.

The protective haplotypes (DR15)-DQB1*0602, (DR5)-DQA1*05-DQB1*0301 and (DR1301)-DQB1*0603 were also used. Individuals carrying different genotypes were graded according to risk of T1D, see Table 30.

Insulin gene and polymorphisms: This assay was performed in the same way as in Paper II, with the polymorphism detected as -23 *HphI* A/T restriction site polymorphism.

Statistical methods

Standard curves and interpolated values of samples were performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, California, USA). For statistical analyses, the statistical package SPSS 11.0 (SPSS Inc., Chicago, Illinois, USA) was used.

The levels of autoantibodies were not normally distributed and therefore non-parametric tests or tests for categorical variables were used.

Comparisons between three or more unpaired groups were analysed with Kruskal Wallis test and comparisons between two unpaired groups with Mann Whitney's U-test. Comparisons between paired groups were analysed with Wilcoxon signed rank test. Correlations were analysed with Spearman's rank correlation coefficient test. A two-tailed p value of 0.05 or less was considered statistically significant.

The Chi-square test was used for categorical variables. A p value of 0.05 or less was interpreted as a significant association. Chi-square with Yates' correction was used for all 2x2 tables in Paper II. In Paper III and IV, Yates' correction were only used for 2x2 tables with expected count < 5 in one cell or more, but a minimum expected count of 1. In paper III: categorical variables significant by the Chi-square tests were analysed by logistic regressions.

Logistic regression analysis is used to explore predictors and to calculate odds ratios (OR) with 95% confidence intervals (CI) for binary dependent variables. An OR above 1.0 indicates increased risk, but only if the CI does not include 1.0 or is too large. The logistic regression analyses (enter) were used when estimating the OR for binary variables, significant in the univariate (Chi-square) analyses, in relation to the dependent variable (beta-cell autoimmunity). If an external factor indicated increased risk of beta-cell autoimmunity in the univariate analyses, it was analysed

simultaneously and included in a multivariate model and selection of significant explanatory variable by logistic regression analyses (forward stepwise).

A p value given as 0.000 by the SPSS program is reported here as $p < 0.001$. No corrections were made for multiple comparisons.

Ethical considerations

Paper I: Diabetes-related autoantibodies in non-diabetic children

The protocol for the prospective prophylaxis study was approved by the Human Research Ethics committee of the Medical Faculty, Linköping University. All parents-to-be gave their written informed consent to enter the study.

The families were recruited from 10 maternity clinics in the Linköping area, and midwives asked pregnant woman about their own and family members' history of allergic disease/atopy. The midwives informed the eligible woman about the purpose of the study. More information about the study as well as verification of the preliminary diagnosis was given over the telephone by the Paediatrician in charge of the study. All mothers allocated to the diet-group were informed that they were welcome to continue with the follow-up of the baby even if they did not adhere to the diet restrictions.

Paper II: Swedish and Lithuanian children with type 1 diabetes

The protocol for the DEBS study was approved by the Research Ethics Committees of the Medical Faculties of Linköping University and Lund University in Sweden and by the Research Ethics Committee of Kaunas University of Medicine in Lithuania. All newly diagnosed patients were invited to participate in the study. All participants and parents/guardians gave their written informed consent to enter the study.

Paper III: Breast-feeding & autoantibodies in children from the general population

The protocol for the ABIS study was approved by the Research Ethics Committees of the Medical Faculty of Linköping University, Linköping and of the Medical Faculty of Lund University, Lund. Parents/guardians gave their informed consent to enter the study.

Before entering the ABIS study all parents-to-be received both written and oral information before the baby was born. They were also offered to watch a video-film about the ABIS study. Return of completed at-birth questionnaire and/or any

biological samples (in addition to cord blood) was considered as informed consent. Likewise, participation in the 1 year, 2-3 year and 5-6 year follow-up with return of completed questionnaires and/or any biological samples was considered as additional informed consent from the parents. The children in the study are identified by an ABIS number only. The data from questionnaires and laboratory analysis are stored without personal identifications and the data is stored and handled with due confidentiality.

Ethical questions in the ABIS study has been studied separately (Stolt *et al.*, 2002) as well as the maternal attitude to participation (Ludvigsson *et al.*, 2001).

Paper IV: Risk genes & autoantibodies in children from the general population

For ethical considerations of the ABIS study, see Paper III.

RESULTS & DISCUSSION

Paper I: Diabetes-related autoantibodies in non-diabetic children

In non-diabetic children with allergic heredity we found low levels of beta-cell autoantibodies with some fluctuations. We defined low levels of GADA, IA-2A and IAA as levels above the detection limit, i.e. the lowest standard. Samples with levels lower than the detection limit were given an arbitrary value.

Prevalence & fluctuation of beta-cell autoantibodies

GADA were detectable in almost all children, IA-2A in about half and IAA in about 10% of all children. The prevalence of detectable GADA, IA-2A and IAA are shown in Table 3.

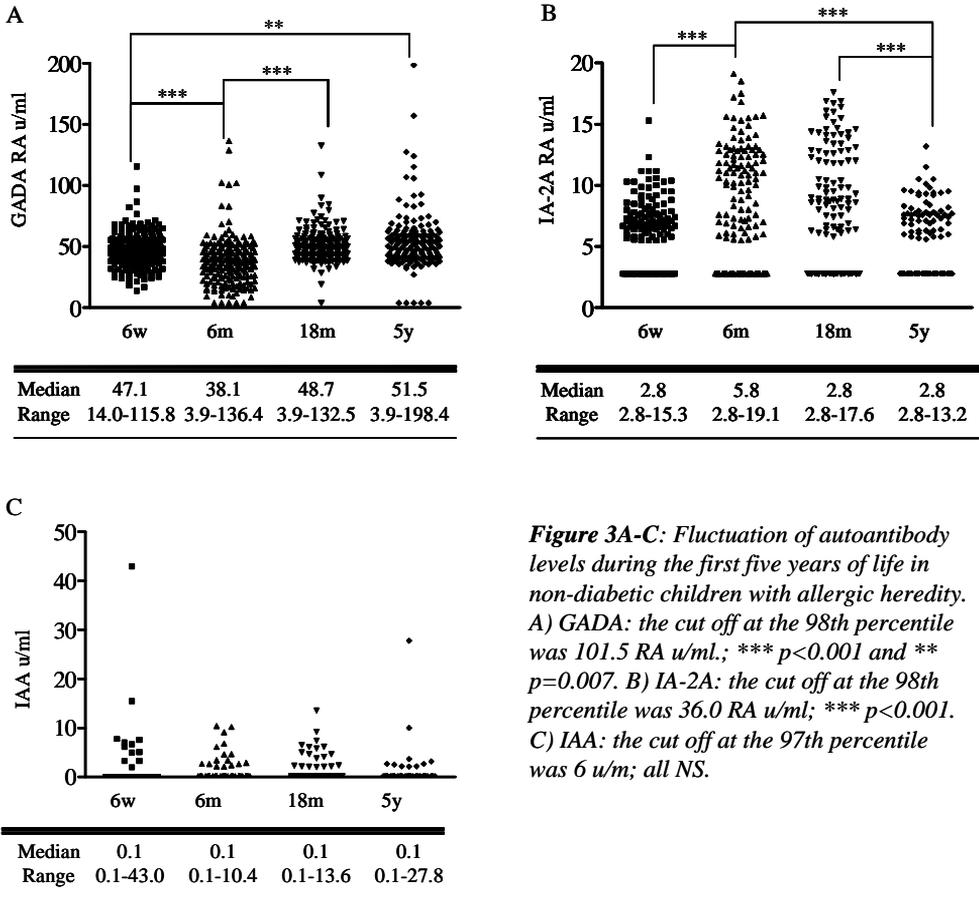
Table 3: The prevalence of detectable GADA, IA-2A and IAA below the cut off limit for positivity in non-diabetic children with allergic heredity.

	GADA N (%)	IA-2A N (%)	IAA N (%)
6 w	196/196 (100.0)	92/196 (46.9)	12/196 (6.1)
6 m	186/191 (97.3)	97/186 (52.2)	16/186 (8.6)
18 m	184/185 (99.5)	81/183 (44.3)	19/180 (10.6)
5 y	152/157 (96.8)	61/156 (39.1)	10/154 (6.5)

When using the cut off limits for positivity 1/196 (0.5%) of the children were positive for GADA at 6 weeks of age, 4/191 (2.1%) at 6 months of age, 2/185 (1.1%) at 18 months of age and 7/157 (4.5%) at 5 years of age. For IAA 7/196 (3.6%) of the children were positive at 6 weeks of age, 5/186 (2.7%) at 6 months of age, 6/180 (3.3%) at 18 months of age and 2/154 (1.3%) at 5 years of age. No children were positive for IA-2A. Only one child was double positive, for both GADA and IAA at 6 months of age, but the level of both autoantibodies decreased at 18 months and at 5 years of age.

We compared the levels of each autoantibody at different time points with Wilcoxon test. The levels of GADA, IA-2A and IAA fluctuated during the first 5 years of life in non-diabetic children. The level of GADA decreased from 6 weeks to 6 months of age ($p < 0.001$), increased between the age of 6 and 18 months ($p < 0.001$) and then remained similar at the age of 5 years (Figure 3A). The levels of IA-2A increased from 6 weeks to 6 months of age ($p < 0.001$) but then decreased until the age of 5 years ($p < 0.001$)

(Figure 3B). No significant changes in the levels of IAA were found, presumably due to the low prevalence of detectable IAA (Figure 3C).



At the age of 6 weeks, we detected a weak correlation between the levels of GADA and IA-2A ($\rho = 0.18$, $p = 0.01$). Also at the age of 6 months we found a weak correlation between the levels of GADA and IA-2A ($\rho = 0.25$, $p = 0.001$) as well as between the levels of GADA and IAA ($\rho = 0.26$, $p < 0.001$). At 5 years of age we found a weak correlation between IA-2A and IAA ($\rho = 0.26$, $p = 0.001$).

Dietary factors & beta-cell autoantibodies

Maternal elimination of cow milk and egg from the diet during the last trimester of pregnancy did not influence neither the prevalence of detectable autoantibodies nor the

levels of autoantibodies in the children at any age (data not shown) (Ludvigsson, 2003).

The median duration of total breastfeeding was 7 months, and the duration of total breastfeeding did not influence the level or prevalence of detectable GADA, IA-2A or IAA in the children (data not shown). Children who were exposed to cow milk before 6 months but after 3 months of age had higher levels of GADA at 6 months than children who had not been exposed to cow milk (median levels of GADA was 39.6 and 32.4 RA u/ml respectively, $p=0.029$, Mann-Whitney test). No associations of cow milk exposure before 6 months of age and IA-2A or IAA were found (data not shown) (Ludvigsson, 2003).

Ear infection or atopy & beta-cell autoantibodies

The levels of IA-2A at 18 months of age were higher in children who had otitis media (middle ear infection) before 6 months of age than in children with no otitis (median levels of IA-2A was 2.8 and 6.3 RA u/ml respectively, $p=0.048$, Mann-Whitney test). Children diagnosed as having a atopic disease at 18 months of age or earlier had lower levels of IA-2A at 5 years of age than children without atopy (median levels of IA-2A was 2.8 and 6.2 RA u/ml, $p=0.002$, Mann-Whitney test) (Ludvigsson, 2003).

Discussion

We found low but clearly detectable levels of beta-cell autoantibodies in non-diabetic children with allergic heredity. The levels are far below the cut off limits which are used to define risk of T1D. Even so, we believe that the autoantibodies detected here are antigen-specific because the binding was effectively inhibited by homologous antigen (data not shown).

GADA, IA-2A and IAA showed different patterns. Production of IA-2A started already at 6 months of age and the levels declined after 18 months of age which may be interpreted as development of tolerance. The level of GADA first decreased from 6 weeks to 6 months, indicating a clearance of maternal GADA. The children's own production of GADA started later, and reached a plateau at 18 months, where the level remained the same at 5 years of age. This is in line with previous results reporting increasing levels of GADA with age and increase in the prevalence of GADA with age in newly diagnosed patients with T1D, opposed to the prevalence of IA-2A and IAA which decreases with age in T1D (Vardi *et al.*, 1988; Verge, Howard, Rowley *et al.*, 1994). In the case of IAA no pattern can be seen since IAA was detected in fewer children. This may to some extent be explained by the low sensitivity of our method. IAA is often reported to be the first autoantibody to appear in the pre-diabetes stage of high risk individuals (Ziegler *et al.*, 1999; T. Kimpimäki *et al.*, 2001; Kukko *et al.*, 2005). The autoantibodies detected at 6 weeks of age probably reflect transfer of

maternal antibodies via the placenta, since IgG antibodies cross the placenta especially during the third trimester as reviewed in (Simister, 2003). Autoantibodies in cord blood has been shown to reflect the autoantibody status of the mother (Ziegler *et al.*, 1993; Hämäläinen *et al.*, 2002). The level of IgG in infants decrease until 3-6 months of age when the levels starts to increase due to the child's own production of IgG (Fulginiti *et al.*, 1966).

The different pattern of fluctuations for GADA and IA-2A may reflect different types of immune response against GAD₆₅ and IA-2 and/or different mechanisms of development of tolerance. Autoantibodies to GAD₆₅ is not a phenomenon restricted to T1D, but are common also in other autoimmune conditions, such as in Stiff-Mans Syndrome (WHO, 1999). IA-2A have a good predictive value for T1D in children as shown by several studies, and could be considered more specific for T1D.

The risk of T1D conferred by positivity to a single beta-cell autoantibody is small, instead multiple positivity confers an increased risk of developing T1D (Bingley *et al.*, 1994; Verge *et al.*, 1998). In the children of our study, the low levels of autoantibodies indicate a low risk of progression to T1D. Accordingly, none of the children still living in the Linköping region has been diagnosed with T1D.

Maternal abstention from cow milk and egg during the last trimester of pregnancy did not influence the level of autoantibodies, nor did the length of total breastfeeding. However, exposure to cow milk by 6 months of age was associated with higher levels of GADA at 6 months of age. According to the study design, the children received casein hydrolysate until three months of age as supplementary feeding. By this age the gut maturation is well underway in humans and we could not evaluate the effect of early exposure (i.e. before 2-4 months of age) to cow milk on the levels of autoantibodies in the present study. Within the original study, the maternal abstention from cow milk was not found to protect the children from developing allergies (Fälth-Magnusson & Kjellman, 1987).

Children diagnosed with atopic disease at 18 months had lower levels of IA-2A at 5 years of age than children without atopic diseases. It is possible that development of atopy and a TH2 deviated immune response has inhibitory effect on the development of IgG-class autoantibodies. Children with ear infections before 6 months of age had increased levels of IA-2A at 18 months when compared to children without ear infections. This might be due to an infection-mediated triggering of the immune system and the trigger is likely non-antigen specific.

Our population is not a randomly selected population of healthy individuals, as all the children have an increased risk to develop atopic disease or allergy. A deviation towards a type 2 immune response characterise atopic disease and allergy and T1D is generally considered as a deviation towards a type 1 response. If this is the case, there

is no reason why atopic children should be more prone to develop autoantibodies. However, both atopic disease and T1D may be regarded as diseases with an aberrant immune regulation/function and it may therefore not be possible to apply our findings to a healthy population.

In summary, our results demonstrate that low levels of autoantibodies against GAD₆₅, IA-2 and insulin are detectable in most children without increased risk of T1D and that the levels of these autoantibodies fluctuate over the first 5 years of life. Our findings suggest that immune response to IA-2 is induced during the first months of life resulting in tolerance. Immune response to GAD₆₅ seems to be induced later and the levels of GADA increase with age in non-diabetic children.

Paper II: Swedish and Lithuanian children with type 1 diabetes

We compared newly diagnosed patients from Sweden and Lithuania to describe differences in beta-cell autoimmunity, T1D associated risk genes and clinical manifestation. Since the incidence of T1D is much higher in Sweden than in Lithuania, we hypothesised that the process leading to T1D may differ and thereby also the prevalence of beta-cell autoantibodies and risk genes. In this study we used the 98th percentile as cut off for autoantibody positivity.

Beta-cell autoantibodies in patients and family members

We detected IAA above the cut off for positivity in 53/96 (55.2%) Swedish and 38/96 (39.6%) Lithuanian patients ($p=0.043$) and GADA in 73/96 (76.0%) Swedish and 57/96 (59.4%) Lithuanian patients ($p=0.032$) (Table 4). The prevalence of IA-2A in Swedish 61/96 (63.5%) and Lithuanian patients 56/96 (58.3%) was similar ($p=0.6$). The levels of IAA in IAA positive patients were similar in Sweden and Lithuania. The levels of GADA in GADA positive patients were higher in Lithuania ($p=0.005$), and the levels of IA-2A in IA-2A positive patients were higher in Sweden ($p=0.005$) (Table 4). The frequencies of family members positive for any of the autoantibodies were small and no statistical differences could be detected (Table 4).

Table 4: Prevalence of beta-cell autoantibodies and level of autoantibodies in patients, parents and siblings in Sweden and Lithuania. Data are the number of subjects positive/total number of subjects (%), range and median level of autoantibody. Levels of IAA are given in u/ml and levels of GADA and IA-2A in RA u/ml. Frequency of autoantibodies were compared by Chi-square with Yates' correction and comparisons of median levels of autoantibodies between countries with Mann-Whitney test.

	IAA		GADA		IA-2A	
	Sweden	Lithuania	Sweden	Lithuania	Sweden	Lithuania
Patients	53/96 (55.2) ^a	38/96 (39.6) ^a	73/96 (76.0) ^b	57/96 (59.4) ^b	61/96 (63.5)	56/96 (58.3)
Median	20.2	17.4	397.0 ^c	1600.0 ^c	2079.0 ^d	420.0 ^d
(range)	(6.8-272.8)	(6.5-1333.0)	(105.6-88*10 ⁶)	(115.7-219450)	(35.5-34740)	(32.9-35200)
Parents	7/172 (4.1)	9/167 (5.4)	10/172 (5.8)	7/167 (4.2)	0/172	2/167 (1.2)
Median	14.0	11.1	334.3	352.8		
(range)	(11.7-299.2)	(7.4-251.2)	(113.2-44000)	(178.0-403000)		
Siblings	3/71 (4.2)	5/81 (6.2)	4/71 (5.6)	2/81 (2.5)	2/71 (2.8)	1/81 (1.2)
Median	13.0	13.0	126.5	1350.0		
(range)	(12.3-112.4)	(6.8-31.6)	(112.7-182.8)	(1000.0-1700.0)		

^a $p=0.043$, ^b $p=0.032$, ^c $p=0.005$ and ^d $p=0.005$, all other p values were non significant

In the entire patient population, the level of IAA did not correlate with the levels of IA-2A ($\rho=-0.051$, $p=0.7$) or the level of GADA ($\rho=0.004$, $p=1.0$) and the level of GADA did not correlate with the level of IA-2A ($\rho=-0.082$, $p=0.5$).

The prevalence of multiple autoantibodies (2 or 3 autoantibodies) was higher in Swedish patients, and the number of autoantibody negative patients were higher in Lithuania ($p=0.015$) (Table 5).

Table 5: The prevalence of positivity for multiple beta-cell autoantibodies in Swedish and Lithuanian patients. Frequency of autoantibodies were compared by Chi-square test.

Autoantibody status	Sweden N (%)	Lithuania N (%)	p-value	Overall p-value
Positive all three	26/96 (27.1)	13/96 (13.5)	0.031	
Positive any two	44/96 (45.8)	43/96 (44.8)	1.000	
Negative all three	5/96 (5.2)	14/96 (14.6)	0.053	0.015

Patient age and gender & beta-cell autoantibodies

The median age in both Swedish and Lithuanian patients was 9.0 years. The patients were divided into three age groups; 1-4 years, 5-9 years and 10-15 years (Sadauskaite-Kuehne *et al.*, 2002). In Lithuania IAA were more prevalent in children below 5 years of age compared to older children ($p=0.001$), this was not seen in Swedish patients ($p=0.5$) (Table 6). There was an inverse correlation between the level of IAA in IAA positive patients and the age of the patient in the Swedish patient group ($\rho=-0.613$, $p<0.001$), but no significant correlation was found in Lithuanian patients ($\rho=-0.242$, $p=0.1$).

Table 6: The distribution of IAA positive children in different age groups in Swedish and Lithuanian patients. Frequencies of autoantibodies were compared by Chi-square tests.

	1-4 years N (%)	5-9 years N (%)	10-15 years N (%)	p value
Sweden	7/10 (70.0)	25/44 (56.8)	21/41 (51.2)	0.553
Lithuania	11/14 (78.6)	8/35 (22.8)	19/47 (40.4)	0.001

Gender had no influence on the level of IAA in IAA positive patients (data not shown). Age and gender did not affect GADA levels (data not shown).

The prevalence of IA-2A was similar in the three age groups in both Sweden and Lithuania (data not shown). There was a weak correlation between the level of IA-2A in IA-2A positive patients and patient age in the entire patient population ($\rho=0.187$, $p=0.044$). This correlation was not significant in the separate patient groups (data not shown). The level of IA-2A was higher in the oldest age group (1-4 years old; median 241.9 range 35.5-8060.0, 5-9 years old; median 1037.0, range 44.9-35 200.0 and 10-15 years old; median 2079.0, range 32.9-34 740.0, $p=0.047$). Gender did not influence the level of IA-2A (data not shown).

Parent's age and autoimmunity & beta-cell autoantibodies in the patients

The age of the mother of Swedish patients (median 37.0 years) was similar to that of the mothers of Lithuanian patients (median 37.0 years) ($p=0.4$). Similarly, the age of Swedish fathers (median 39.0 years) was similar to that of Lithuanian fathers (median 36.5 years) ($p=0.3$). The age of the mother or father did not affect the levels of IAA, GADA or IA-2A in the patients (data not shown). Similarly, autoimmune diseases (T1D, rheumatic disease, celiac disease or thyroid disease) or type 2 diabetes (T2D) did not affect the levels of IAA, GADA or IA-2A in the patients (data not shown). This may be explained by the low numbers of parents with these diseases: 12 reported T1D (10 fathers and 2 mothers), one reported T2D, 5 reported rheumatic disease, 2 reported celiac disease and 9 reported thyroid disease.

Beta-cell autoantibodies & clinical characteristics

The IAA negative patients showed signs of a more serious clinical manifestation at onset of diabetes, independently of the age. To be able to stratify for age, the patients from Sweden and Lithuania were analysed together in relation to clinical parameters. In the entire patient population, the HbA1c level was elevated in IAA negative patients (median 12.4, range 6.1-14.5) compared to in IAA positive patients (median 9.8, range 5.7-14.0) ($p=0.001$). Among patients positive for IAA, HbA1c correlated inversely with the level of IAA ($\rho=-0.339$, $p=0.007$). Similarly, the level of ketones was elevated in the IAA negative patients (median 3, range 0-4) compared to in IAA positive (median 2, range 0-4) ($p=0.006$). In patients positive for IAA, the levels of ketones correlated inversely with the level of IAA ($\rho=-0.300$, $p=0.012$). The findings when analysing Swedish and Lithuanian patients separately are given in Table 7. The association of negative IAA with HbA1c and ketones were similar in the age groups 1-4 years and 5-9 years, although no differences were seen in the age group 10-15 years (Table 8). There were no associations between GADA and signs of a more serious clinical manifestation at onset of diabetes (data not shown). In patients positive for IA-2A, blood glucose levels correlated positively with the level of IA-2A ($\rho=0.275$, $p=0.003$). The level of ketones was significantly lower in patients positive for all three autoantibodies (median 2, range 0-4) compared to patients with two autoantibodies (median 3, range 0-4) or no autoantibodies (median 3, range 0-4) ($p=0.029$). The levels

of HbA1c, blood glucose and pH values in the patients were not influenced by prevalence of two or three different autoantibodies, or by the absence of autoantibodies (data not shown).

Table 7: The clinical characteristics (HbA1c and ketones) in Swedish and Lithuanian patients at diagnosis. Comparisons were made by the Mann-Whitney test and the Spearman's rank correlation test.

			Median	Range	N	p value	(rho), p value
Swedish	HbA1c	IAA pos	9.0	5.7-13.4	30	0.2	(-0.3), 0.1
		IAA neg	9.5	6.1-14.1	25		
	Ketones	IAA pos	1	0-3	31	0.1	(-0.4), 0.034
		IAA neg	2	0-3	24		
			Median	Range	N	p value	(rho), p value
Lithuanian	HbA1c	IAA pos	10.3	6.7-14.0	33	0.017	(-0.4), 0.02
		IAA neg	12.4	7.0-14.5	52		
	Ketones	IAA pos	3	0-4	38	0.2	(0.2), 0.1
		IAA neg	3	0-4	58		

Beta-cell autoantibodies & genetic risk of type 1 diabetes

The risk haplotype HLA-DR4-DQ8 (DQB1*0302) was more common among Swedish than Lithuanian patients, 65/81 (80.2%) of Swedish and 52/86 (60.5%) of Lithuanian patients carried this haplotype ($p=0.004$). The risk haplotype DR3-DQ2 (DQA1*05-DQB1*02) was detected in 43/81 (53.1%) of Swedish and 37/86 (43.0%) of Lithuanian patients (NS). The high-risk combination of these both haplotypes was also more frequent among Swedish 32/81, (39.5%) than Lithuanian 17/86 (19.8%) patients ($p=0.009$). In the entire patient population, the number of IAA or GADA positive children did not differ between children with and without DR4-DQ8 or DR3-DQ2 haplotype (Table 9). The IA-2A positivity was associated with DR4-DQ8 haplotype in patients ($p<0.001$), but not with the DR3-DQ2 haplotype (Table 9).

RESULTS & DISCUSSION

Table 8: The clinical characteristics (HbA1c and ketones) in the entire patient population, stratified for age. Comparisons were made by the Mann-Whitney test and the Spearman's rank correlation test.

			Median	Range	N	p value	(rho), p value
1-4 years	HbA1c	IAA pos	9.4	5.7-12.3	15	0.2	(-0.645), 0.009
		IAA neg	10.7	7.0-11.5	4		
	Ketones	IAA pos	2	0-4	15	1.0	(-0.515), 0.049
		IAA neg	2	0-4	4		
			Median	Range	N	p value	(rho), p value
5-9 years	HbA1c	IAA pos	9.5	6.2-14.0	22	0.019	(-0.460), 0.031
		IAA neg	11.1	6.1-14.3	38		
	Ketones	IAA pos	2	0-4	24	0.069	(-0.580), 0.003
		IAA neg	3	0-4	40		
			Median	Range	N	p value	(rho), p value
10-15 years	HbA1c	IAA pos	10.5	7.1-14.0	26	0.2	(0.090), 0.7
		IAA neg	12.4	8.2-14.5	35		
	Ketones	IAA pos	3	0-4	30	0.034	(-0.003); 1.0
		IAA neg	3	0-4	38		

Table 9: Prevalence of autoantibodies in the entire patient population with or without HLA risk haplotypes. Frequencies were compared by Chi-square test with Yates' correction.

Haplotype		IAA positive N (%)	GADA positive N (%)	IA-2A positive N (%)
DR4-DQ8	Yes	59/116 (50.8)	72/112 (64.3)	81/114 (71.1)
	No	22/52 (42.3)	40/52 (76.9)	21/52 (40.4)
	p value	0.4	0.2	<0.001
DR3-DQ2	Yes	41/80 (51.3)	55/78 (70.5)	44/78 (56.4)
	No	40/87 (46.0)	56/111 (50.5)	57/87 (65.6)
	p value	0.6	0.6	0.3
DR4-DQ8 & DR3-DQ2	Yes	28/49 (57.1)	30/47 (63.8)	32/47 (68.1)
	No	53/65 (81.5)	81/116 (69.8)	69/118 (58.5)
	p value	0.2	0.6	0.3

In patients matched according to their DR4-DQ8 or DR3-DQ2 haplotype, the number of GADA positive children was significantly higher in Sweden than in Lithuania (Table 10A). The number of IAA positive or IA-2A positive children did not differ between patients with and without these haplotypes.

The polymorphism in the insulin gene was analysed as -2221 C/T or -23 A/T polymorphism, and the distributions of the genotypes was similar in Swedish and Lithuanian patients (Table 11). The polymorphism in the CTLA-4 gene, analysed as A/G polymorphism, did not differ between Swedish and Lithuanian patients (Table 12). The frequency of IAA, GADA or IA-2A positive children did not differ between patients carrying different genotypes of the insulin gene (Table 13). In patients carrying the CC genotype of the -2221 C/T polymorphism or the AA genotype of the -23 A/T polymorphism, the number of patients positive for IAA was higher in Swedish patients than in Lithuanian patients ($p=0.017$ and $p=0.031$ respectively, see Table 10B). The number of IAA, GADA or IA-2A positive children did not differ between children carrying different CTLA-4 genotypes (Table 14). In patients carrying the GG or AG genotype, the number of patients positive for GADA was higher in Swedish than in Lithuanian patients ($p=0.038$, Table 10C).

Table 10: Beta-cell autoantibodies in Swedish and Lithuanian patients matched according to A) DR4-DQ8 or DR3-DQ2, B) INS VNTR C/C or A/A and C) CTLA-4 G/G and A/G genotypes. Data are the number of patients positive/total number of patients (%). Frequencies were compared by Chi-square test with Yates' correction.

A

		DR4-DQ8 genotype		p value	DR3-DQ2 genotype		p value
		Sweden	Lithuania		Sweden	Lithuania	
IAA	pos	38/65 (58.5)	21/51 (41.2)	0.097	25/43 (58.1)	16/37 (43.2)	0.3
IAA	neg	27/65 (41.5)	30/51 (58.8)		18/43 (41.9)	21/37 (56.8)	
GADA	pos	48/64 (75.0)	24/48 (50.0)	0.011	35/42 (83.3)	20/36 (55.6)	0.015
GADA	neg	16/64 (25.0)	24/48 (50.0)		7/42 (16.7)	16/36 (44.4)	
IA-2A	pos	48/64 (75.0)	33/50 (66.0)	0.4	26/42 (61.9)	18/36 (50.0)	0.4
IA-2A	neg	16/64 (25.0)	17/50 (34.0)		16/42 (38.1)	18/36 (50.0)	

Continues on next page

Table 10: *Continued.*

B

		INS VNTR; 2221 C/C		p value	INS VNTR; 23 A/A		p value
		Sweden	Lithuania		Sweden	Lithuania	
IAA	pos	36/58 (62.1)	27/69 (39.1)	0.017	32/52 (61.5)	24/61 (39.3)	0.031
IAA	neg	22/58 (37.9)	42/69 (60.9)		20/52 (38.5)	37/61 (60.7)	
GADA	pos	42/57 (73.7)	41/66 (62.1)	0.2	37/51 (72.5)	35/58 (60.3)	0.3
GADA	neg	15/57 (26.3)	25/66 (37.9)		14/51 (27.5)	23/58 (39.7)	
IA-2A	pos	36/57 (63.2)	37/68 (54.4)	0.4	33/51 (64.7)	31/60 (51.7)	0.2
IA-2A	neg	21/57 (36.8)	31/68 (45.6)		18/51 (35.3)	29/60 (48.3)	

C

		CTLA-4; G/G and A/G		p value
		Sweden	Lithuania	
IAA	pos	29/52 (55.8)	24/64 (37.5)	0.076
IAA	neg	23/52 (44.2)	40/64 (62.5)	
GADA	pos	42/51 (82.3)	39/62 (62.9)	0.038
GADA	neg	9/51 (17.6)	23/62 (37.1)	
IA-2A	pos	34/51 (66.7)	37/63 (58.7)	0.5
IA-2A	neg	17/51 (33.3)	26/63 (41.3)	

Table 11: *Frequencies of INS-2221 and INS-23 polymorphisms in Swedish and Lithuanian patients. CC and AA genotypes confer risk of T1D, CT, AT and TT genotypes confer varying degrees of protection. Frequencies were compared by Chi-square test with Yates' correction.*

Genotype		Sweden	Lithuania	p value
		N (%)	N (%)	
INS-2221	CC	58/81 (71.6)	69/87 (79.3)	0.5
	CT	21/81 (25.9)	16/87 (18.4)	
	TT	2/81 (2.5)	2/87 (2.3)	
INS-23	AA	52/79 (65.8)	61/85 (71.8)	0.7
	AT	24/79 (30.4)	22/85 (25.9)	
	TT	3/79 (3.8)	2/85 (2.3)	

Table 12: Frequencies of CTLA-4 A/G polymorphism in Swedish and Lithuanian patients. GG and AG genotypes confer risk of T1D, AA genotype confers protection. Frequencies were compared by Chi-square test with Yates' correction.

CTLA-4 A/G genotype	Sweden N (%)	Lithuania N (%)
GG	21/81 (25.9)	24/87 (27.6)
AG	31/81 (38.3)	40/87 (46.0)
AA	29/81 (35.8)	23/87 (26.4)
p value		0.4

Table 13: Prevalence of autoantibodies in relation to INS polymorphism analysed as INS-2221 or INS-23 in the entire patient population. Frequencies were compared by Chi-square test with Yates' correction.

Polymorphism	Genotype	IAA positive N (%)	GADA positive N (%)	IA-2A positive N (%)
INS-2221	CC	63/81 (77.8)	83/112 (74.1)	73/102 (71.6)
	CT	18/81 (22.2)	28/112 (25.0)	26/102 (25.5)
	TT	0	1/112 (0.9)	3/102 (2.9)
p value		0.1	0.1	0.4
INS-23	AA	56/79 (70.9)	72/109 (66.1)	64/98 (65.3)
	AT	23/79 (29.1)	35/109 (32.1)	30/98 (30.6)
	TT	0	2/109 (1.8)	4/98 (4.1)
p value		0.1	0.2	0.5

Table 14: Prevalence of autoantibodies in relation to CTLA-4 polymorphism in the entire patient population. Frequencies were compared by Chi-square test with Yates' correction.

CTLA-4 Genotype	IAA positive N (%)	GADA positive N (%)	IA-2A positive N (%)
GG	25/81 (30.9)	33/112 (29.5)	27/102 (26.5)
AG	28/81 (34.6)	48/112 (42.9)	44/102 (43.1)
AA	28/81 (34.6)	31/112 (27.7)	31/102 (30.4)
p value	0.1	0.3	0.9

Discussion

We found differences in humoral beta-cell autoimmunity and HLA haplotypes between children with newly diagnosed T1D in Sweden and Lithuania. The frequencies of IAA and GADA were higher in Swedish compared to Lithuanian patients. Also, the frequencies of HLA haplotype DR4-DQ8 and the high risk combination of DR4-DQ8 and DR3-DQ2 were higher in Swedish compared to Lithuanian patients. The differences in IAA and GADA frequencies did not diminish when the patients were matched for HLA, insulin or CTLA-4 genotypes. Interestingly, the difference in the prevalence of IAA between Swedish and Lithuanian patients was more significant among patients with the insulin gene risk genotypes. Therefore, the autoimmune response towards insulin and GAD₆₅ seems to be more pronounced in an area with a high incidence of T1D and this pronounced autoimmune response can not be fully explained by the found differences in some risk genes.

As reported previously (Arslanian *et al.*, 1985; Karjalainen *et al.*, 1986; Vardi *et al.*, 1988), the prevalence of IAA was highest in the youngest group of patients. The incidence of T1D has increased in the young children in Sweden during the last decades (Pundziute-Lyckå *et al.*, 2002). This increase may be associated with changes in environment favouring the development of beta-cell autoimmunity or an earlier precipitation of T1D, where IAA and GADA may be specifically associated with this recent increase in T1D.

No differences in the prevalence of autoantibodies were seen among family members in Sweden and Lithuania. The prevalence of beta-cell autoantibodies in first degree relatives in European countries have previously been reported to be similar, despite different incidence of T1D (Williams *et al.*, 2002). The disposition to autoimmunity seems to be similar in Sweden and Lithuania, which is in line with the finding of similar frequency of autoimmune diseases in this study population (Samuelsson *et al.*, 2004). It should be emphasized that the number of family members studied is not large and the prevalence of autoantibodies in family members is low, which limits the interpretation of the data.

We analysed the prevalence and level of beta-cell autoantibodies in relation to clinical characteristics at diagnosis. We found that patients with low levels of IAA had higher levels of HbA1c (Sabbah *et al.*, 1999). When the material was stratified for age, this was true in age groups 1-4 years and 5-9 years, although not in children 10-15 years old. An inverse correlation was found between the levels of IAA and of ketones, and this was seen in all age groups when stratified for age. We did not find any association between IA2-A or GADA positivity and any clinical parameters in accordance with other studies (Komulainen *et al.*, 1997; Sabbah *et al.*, 1999). However, we found that levels of IA-2A among the IA-2A positives correlated with blood glucose levels, suggesting that autoimmunity to IA2-A may indicate poor insulin response in patients.

Our results suggest that patients without IAA have a more severe onset of T1D which may be interpreted as less residual insulin induces less autoantibody response, although the results need to be interpreted with some care due to the multiple comparisons made.

Several studies have shown that multiple autoantibodies increase the risk of T1D in first degree relatives (Bingley *et al.*, 1994; Bingley, 1996; Gardner *et al.*, 1999). The prevalence of patients positive for all three autoantibodies was higher in Sweden than in Lithuania, which is to be expected because the frequencies of both IAA and GADA were higher among the Swedish patients.

The levels of HbA1c, blood glucose and pH were not influenced by prevalence of none or two or three different autoantibodies (Sabbah *et al.*, 1999). However, the level of ketones was significantly lower in patients with all three autoantibodies, supporting our interpretation that the presence of beta-cell autoimmunity in patients may be associated with less aggressive destruction of beta-cells. Increased awareness of T1D in Sweden may lead to an earlier diagnosis and hence less severity at onset as compared to Lithuanian patients (Sadauskaite-Kuehne *et al.*, 2002).

We found a higher frequency of Swedish patients with the high risk HLA genotype combination HLA DR4-DQ8 and DR3-DQ2 compared to Lithuanian patients. Similarly, the frequency of the HLA haplotype DR4-DQ8 was also higher in Swedish patients whereas the frequency of the haplotype DR3-DQ2 was similar in Swedish and Lithuanian patients (Nejentsev *et al.*, 1998). These differences did not explain the differences in prevalence of autoantibodies and neither did the T1D associated polymorphisms in the insulin and CTLA-4 genes. The single nucleotide polymorphisms (SNPs) in the insulin gene, detected as CC-, CT- and TT- genotypes or AA-, AT- and TT- genotypes did not differ between Sweden and Lithuania. The frequencies of the high risk genotypes (CC- and AA- genotypes) and the low risk genotype (TT- genotype) correspond well with earlier studies (Sarugeris *et al.*, 1998; Perez De Nanclares *et al.*, 2003; Walter *et al.*, 2003). The high risk genotypes in the insulin gene polymorphisms were both associated with higher frequency of IAA positive children in Sweden than in Lithuania. The SNP at position 49 in the CTLA-4 gene, detected as AA-, AG- and GG-genotypes, did not differ between Sweden and Lithuania. The high risk genotypes (GG and AG) in the CTLA-4 polymorphism were associated with higher frequency of GADA positive children in Sweden than in Lithuania. We did not perform correction for multiple comparisons since these comparisons were not part of our original hypotheses, but rather originated from our primary findings.

Our results demonstrate that autoantibodies against insulin and GAD₆₅ are more often seen at diagnosis in children with T1D living in Sweden with high incidence of T1D than in Lithuania with low incidence of T1D. The disease phenotype characterized by

the presence of these autoantibodies seems to be clinically milder, although our results are limited to the three major markers of humoral beta-cell autoimmunity. Environmental factors, which protect from autoimmune mediated T1D may dominate in Lithuania while factors precipitating T1D may dominate in Sweden. The autoimmune process may play a more important role in disease development in high incidence areas like Sweden, but additional investigations in larger study populations are required to further elucidate this hypothesis. An alternative explanation is that differences in autoantibodies are due to variation in the progression of the disease process. Our findings favour the view that T1D in children could be divided into subtypes with heterogeneous aetiology.

Paper III: Breast-feeding & autoantibodies in children from the general population

Breast-feeding has been suggested to have a protective effect in the development of T1D. We investigated the association between duration of breast-feeding and development of beta-cell autoimmunity, detected as presence of beta-cell autoantibodies, in children who participated in the prospective population-based ABIS study. The aim was to investigate if duration of breast-feeding is associated with beta-cell autoimmunity in Swedish children from the general population, 5-6 years old. The cut off levels for autoantibody positivity used in this study were the 95th and 99th percentile.

Descriptives

We studied 3788 children with autoantibody analysis and 12 (0.3%) of them developed T1D by the age of 5-6 years and were therefore excluded from further analysis. Among the remaining 3776 non-diabetic children the median duration of total breast-feeding was 8 months and mean duration was 9 months (data available on 2916 children). Median duration of exclusive breast-feeding was 4 months and mean duration was 5 months (data available on 2867 children). At 3 months of age, 2724/2916 (93.4%) of the children were breast-fed and 2491/2867 (86.9%) were exclusively breast-fed. At 9 months of age, nearly half of the children were still receiving breast-milk (Table 15). Sixty-four of 3776 children (1.7%) were positive for at least one of GADA, IA-2A and IAA above the 99th percentile, and 266/3776 children (7.0%) were positive for GADA and/or IAA above the 95th percentile.

Table 15: The number of children breast-fed at different ages among the sub sample of ABIS children where at least one autoantibody was analysed at 5-6 years of age and data on breastfeeding is available. (N=2867 and 2916).

	3 mo N (%)	6 mo N (%)	9 mo N (%)
Total breastfeeding	2724/2916 (93.4)	2378/2916 (81.6)	1406/2916 (48.2)
Exclusive breastfeeding	2491/2867 (86.9)	836/2867 (29.2)	114/2867 (4.0)

Duration of total breast-feeding & beta-cell autoantibodies

In children with a short duration of total breast-feeding, less than 4 months, the prevalence of GADA and/or IAA above the 95th percentile was higher than in children breast-fed longer (14.0% and 7.2% respectively, $p < 0.001$). Similarly, the prevalence of IAA above the 95th percentile was higher in children with duration of total breast-

feeding less than 4 months when compared to those breast-fed longer (12.3% and 4.6% respectively, $p < 0.001$) (Table 16). Short duration of total breast-feeding was associated with an increased risk of GADA and/or IAA above the 95th percentile at 5-6 years of age (OR 2.09; 95% CI 1.45-3.02; $p < 0.001$) as well as with an increased risk of IAA above the 95th percentile at this age (OR 2.89; 95% CI 1.81-4.62; $p < 0.001$) (Table 16).

Table 16: The frequencies of GADA and/or IAA above the 95th percentile and IAA above the 95th percentile in non-diabetic children with short or longer duration of total breast-feeding. Frequencies were compared by Chi-square test and OR were calculated by logistic regression.

Autoantibodies, 95th perc.	Breast-feeding		ChiX	
	0-3 mo; N (%)	≥ 4 mo; N (%)	p value	OR (95% CI); p value
GADA and/or IAA	40/285 (14.0)	179/2469 (7.2)	<0.001	2.09 (1.45-3.02); <0.001
IAA	27/220 (12.3)	70/1515 (4.6)	<0.001	2.89 (1.807-4.615); <0.001

The number of children with GADA, IAA and/or IA-2A above the 99th percentile were not significantly affected by the duration of total breast-feeding (3.0% and 1.6% for 0-3 months vs. 4 months or longer, $p = 0.090$) (Table 17). The numbers of children with IA-2A above the 99th percentile or IAA above the 99th percentile were not affected by the duration of total breast-feeding (Table 17). Similarly, the numbers of children with GADA above the 95th or 99th percentile were not affected by the duration of total breast-feeding (Table 17).

Table 17: Frequencies of single autoantibodies above the 95th or 99th percentile or combination of autoantibodies above the 99th percentile in non-diabetic children with short or longer duration of total breast-feeding. Frequencies were compared by Chi-square test.

Autoantibodies	Breast-feeding		ChiX p value
	0-3 mo; N (%)	≥ 4 mo; N (%)	
GADA, 95th perc.	14/596 (5.4)	115/2277 (5.1)	0.8
GADA, 99th perc.	5/259 (1.9)	21/2277 (0.9)	0.2
IAA, 99th perc.	5/220 (2.3)	14/1515 (0.9)	0.1
IA-2A, 99th perc.	1/279 (0.4)	17/2405 (0.7)	0.7
GADA, IAA, IA-2A, 99th perc.	9/305 (3.0)	42/2614 (1.6)	0.090

Duration of exclusive breast-feeding & beta-cell autoantibodies

In children with a short duration of exclusive breast-feeding, less than 4 months, the prevalence of GADA, IAA and/or IA-2A above the 99th percentile was higher than in children breast-fed longer (2.7 % and 1.4% respectively, $p=0.025$). The prevalence of IA-2A above the 99th percentile was also increased in children exclusively breast-fed less than 4 months when compared to those breast-fed longer (1.6% and 0.5% respectively, $p=0.013$) (Table 18).

Table 18: Frequencies of combination of autoantibodies above the 99th percentile or IA-2A above the 99th percentile in non-diabetic children with short or longer duration of exclusive breast-feeding. Frequencies were compared by Chi-square test and OR were calculated by logistic regression.

Autoantibodies, 99th perc.	Breast-feeding		ChiX	
	1-3 mo; N (%)	≥ 4 mo; N (%)	p value	OR (95% CI); p value
GADA, IA-2A, IA-2A	15 (2.7)	32 (1.4)	0.025	2.01 (1.08-3.73); 0.028
IA-2A	8 (1.6)	10 (0.5)	0.013	3.50 (1.38-8.92); 0.009

The number of children positive for GADA and/or IAA above the 95th percentile was not affected by the duration of exclusive breast-feeding (8.9% and 6.9% for 1-3 months vs. 4 months or longer, $p=0.1$). Neither was the number of children positive for IAA above the 95th percentile not significantly affected by the duration of exclusive breast-feeding (6.5% and 4.2% for 1-3 months vs. 4 months or longer, $p=0.070$). The numbers of children with GADA above the 95th or 99th percentile or IAA above the 99th percentile were not affected by the duration of exclusive breast-feeding (Table 19).

Table 19: Frequencies of combination of autoantibodies above the 95th percentile or single autoantibodies above the 95th or 99th percentile in non-diabetic children with short or longer duration of exclusive breast-feeding. Frequencies were compared by Chi-square test.

Autoantibodies	Breast-feeding		ChiX p value
	1-3 mo; N (%)	≥ 4 mo; N (%)	
GADA, IAA, 95th perc.	46/516 (8.9)	151/2177 (6.9)	0.1
IAA, 95th perc.	22/337 (6.5)	56/1333 (4.2)	0.070
IAA, 99th perc.	4/337 (1.2)	11/1333 (0.9)	0.8
GADA, 95th perc.	26/469 (5.5)	100/2003 (5.0)	0.6
GADA, 99th perc.	8/469 (1.7)	16/2003 (0.8)	0.1

Short duration of exclusive breast-feeding was associated with a risk of GADA, IAA and/or IA-2A above the 99th percentile (OR 2.01; 95% CI 1.08-3.73; $p=0.028$) as well as with a risk of IA-2A above the 99th percentile (OR 3.50; 95% CI 1.38-8.92; $p=0.009$) at 5-6 years of age (Table 18).

Introduction of cow milk proteins & beta-cell autoantibodies

We also investigated if early introduction of cow milk proteins in formula was associated with beta-cell autoantibodies. The duration of exclusive breast-feeding correlated to age of exposure to formula ($\rho=0.620$, $p<0.001$). The majority of the non-diabetic children (1619/2675; 60.5%) received formula for the first time at 5-9 months of age, and 667 children received cow milk free formula (Table 20). In children who received formula early, at 1-3 months of age, the prevalence of GADA, IAA and/or IA-2A above the 99th percentile was increased compared to children who received formula later (2.4% and 1.3% respectively, $p=0.043$) and was associated with a risk of GADA, IAA and/or IA-2A above the 99th percentile (OR 1.84; 95% CI 1.01-3.37; $p=0.047$) (Table 21). In children who received formula early, at 1-3 months of age, the prevalence of IAA above the 95th percentile was not significantly different compared to children who received formula later (6.1% and 4.3% respectively, $p=0.1$) (Table 21).

Table 20: *The number of children who received formula and cow milk free formula for the first time at different ages among the sub sample of ABIS children where at least one autoantibody was analysed at 5-6 years of age and data on formula is available. (N=2675 and 667).*

Age	Formula N (%)	Cows milk free formula N (%)
1-2 mo	635/2675 (23.7)	168/667 (25.2)
3-4 mo	421/2675 (15.7)	85/667 (12.7)
5-9 mo	1619/2675 (60.5)	414/667 (62.1)

External factors & beta-cell autoantibodies

The external factors maternal age, maternal education, infections during pregnancy, mode of delivery, low birth weight (<2500 g), early gestation age (≤ 37 weeks), first degree relative with T1D or celiac disease (CD) or type 2 diabetes (T2D), or gastroenteritis in the child during its first year or recurrent until 5-6 years of age, were considered as potential confounder variables and were tested by Chi-square test. The significant variables were entered as covariates in a multivariate model.

The prevalence of IAA above the 95th percentile was higher in children with a first degree relative with CD compared to those with no CD in the family (12.9% and 4.9% respectively, p=0.044). However, in a forward stepwise logistic regression with IAA as the dependent variable and the duration of total breast-feeding and CD in the family as covariates, CD in the family did not affect the risk mediated by short duration of breast-feeding (Table 22).

Table 21: Introduction of formula in non-diabetic 5-6 year old children with IAA above the 95th percentile or GADA, IAA and/or IA-2A above the 99th percentile. Frequencies were compared by Chi-square test and OR were calculated by logistic regression.

	1-3 mo N (%)	≥ 4 mo N (%)	Total N	ChiX p value	OR (95% CI); p value
IAA, 95th perc.	29/475 (6.1)	47/1083 (4.3)	1558	0.1	
GADA, IAA, IA-2A 99th perc.	19/787 (2.4)	25/1888 (1.3)	2675	0.043	1.84 (1.01-3.37); 0.047

Table 22: The external factor "CD in first degree relative" and IAA above the 95th percentile in 5-6 year-old non-diabetic children. The external factor was tested in forward stepwise logistic regression together with duration of total breast-feeding and IAA above the 95th percentile. The "CD in first degree relative" variable did not enter the forward stepwise logistic regression model, and does not affect the risk mediated by short duration of total breast-feeding.

External factor	IAA 95th perc.
CD in 1st degree relative	4/31 (12.9)
No CD	107/2170 (4.9)
ChiX p value	0.044
FW Log. Reg; OR (95% CI); p value	NE

NE; external factor did not enter the model. CD; celiac disease. FW Log. Reg; forward stepwise logistic regression.

The prevalence of IA-2A above the 99th percentile was higher in those children whose mothers reported having had an infection during pregnancy compared to those with no infection (1.1% and 0.5% respectively, p=0.051) (Table 23) and when the child was delivered via caesarean section compared to vaginal delivery (1.6% and 0.5% respectively, p=0.027) (Table 24). However, in a forward stepwise logistic regression with IA-2A as the dependent variable and the duration of exclusive breast-feeding and

either infection during pregnancy or mode of delivery as covariates, neither of them affected the risk mediated by short duration of breast-feeding (Table 23 and 24).

Table 23: The external factor "Infection during pregnancy" and autoantibodies in 5-6 year-old non-diabetic children. The external factor was tested in forward stepwise logistic regression together with duration of total breast-feeding and GADA and/or IAA above the 95th percentile or exclusive breast-feeding and IA-2A above the 99th percentile. "The Infection during pregnancy" variable did not enter the forward stepwise logistic regression models or was non-significant, and does not affect the risk mediated by short duration of total or exclusive breast-feeding.

External factor	IA-2A 99th perc.	GADA, IAA 95th perc.
Infection during pregnancy; N (%)	9/837 (1.1)	51/856 (6.0)
No infection during pregnancy; N (%)	11/2387 (0.5)	195/2440 (8.0)
ChiX p value	0.051	0.051
FW Log. Reg: OR (95% CI); p value	NE	0.77 (0.55-1.08); 0.1

NE; external factor did not enter the model. FW Log. Reg; forward stepwise logistic regression.

Table 24: The external factor "Mode of delivery" and IA-2A above the 99th percentile in 5-6 year-old non-diabetic children. The external factor was tested in forward stepwise logistic regression together with duration of exclusive breast-feeding and IA-2A above the 99th percentile. The "Mode of delivery" variable did not enter the forward stepwise logistic regression model, and does not affect the risk mediated by short duration of exclusive breast-feeding.

External factor	IA-2A 99th perc.
caesarean section	6/367 (1.6)
vaginal delivery	14/2770 (0.5)
ChiX p value	0.027
FW Log. Reg: OR (95% CI); p value	NE

NE; external factor did not enter the model. FW Log. Reg; forward stepwise logistic regression.

The prevalence of GADA and/or IAA above the 95th percentile tended to increase in those children whose mothers reported not having had an infection during pregnancy compared to those with an infection (8.0% and 6.0% respectively, p=0.051). However,

in a forward stepwise logistic regression with GADA and/or IAA as the dependent variables and the duration of exclusive breast-feeding and infection during pregnancy as covariates, infection during pregnancy did not affect the risk mediated by short duration of breast-feeding (Table 23).

All other abovementioned external factors were found to be non-significant in Chi-square tests with either IAA above the 95th percentile, IA-2A above the 99th percentile, GADA and/or IAA above the 95th percentile or GADA, IAA and/or IA-2A above the 99th percentile, and were therefore excluded from any logistic regression analysis.

Discussion

We found an association between the duration of total breast-feeding and positivity for GADA and/or IAA at the age of 5-6 years in children from the general population, which suggests that breast-feeding has a long term effect on the risk of beta-cell autoimmunity several years after completing breast-feeding. As breast-milk contains growth factors and cytokines it promotes the maturation of the intestinal mucosa and thus the protective effect of breast-feeding might be explained by improved function of the gut immune system as reviewed in (Harrison & Honeyman, 1999). The importance of breast-feeding in the induction of oral tolerance has been indicated in epidemiological studies of celiac disease (CD) (Ivarsson *et al.*, 2002), where the introduction of wheat gluten during the breast-feeding was found to decrease the risk of CD later in life. Interestingly enough, we found a long-term protective effect of breast-feeding or possibly a diabetogenic effect of cow milk formula, on beta-cell autoimmunity in non-diabetic children. An increased risk of autoimmunity associated with CD at 5 years of age has been reported in children exposed to gluten in the first 3 months of life (Norris *et al.*, 2005). This may be interpreted as a slowly escalating immune response by an immature immune system towards a continued exposure to the antigen.

We also found an association between the duration of exclusive breast-feeding and positivity for GADA, IAA and/or IA-2A at the age of 5-6 years. The risk of T1D associated with short duration of exclusive breast-feeding (less than 4 months) may be mediated by a diabetogenic effect of cow milk formula, which is usually used for the weaning, or by the protective effect of breast milk itself. Immunization to bovine insulin in cow milk has been suggested to be the link explaining the cow milk mediated risk of T1D in children with aberrant function of the gut immune system (Vaarala, 1999; Knip *et al.*, 2005). To study the relation of cow milk exposure and development of an autoimmune response to insulin we studied the occurrence of IAA at 5-6 years of age using the 95th percentile as cut off for positivity. It is of special interest that the risk for IAA above the 95th percentile was increased in the children who were breast-fed shorter than 4 months, as this was not found for GADA.

IAA is often the first autoantibody detected in young children (Ziegler *et al.*, 1999) and the prevalence of IAA is highest in the youngest children (Arslanian *et al.*, 1985; Karjalainen *et al.*, 1986; Vardi *et al.*, 1988), suggesting that insulin is an important primary autoantigen in young children who develop T1D. Although the short duration of breast-feeding was associated specifically with IAA indicating a clue to insulin, we observed only a weak association between the occurrence of IAA and the duration of exclusive breast-feeding, which correlates strongly with the age at exposure to cow milk formula in the Swedish population. We did not observe any association between the occurrence of IAA and introduction of formula at the age of 1-3 months. Instead IA-2A above the 99th percentile alone or in combination with GADA and/or IAA above the 99th percentile was associated both with duration of exclusive breast-feeding and with introduction of formula at the age of 1-3 months.

The interaction between the suspected dietary trigger of the disease and the factors which modulate the effect of the trigger is complex in humans. As mentioned earlier, breast-feeding during introduction of wheat gluten has been reported to modify the risk of CD in Swedish studies, but when the risk of CD-related autoimmunity and timing of gluten introduction was studied in a North-American follow-up study, the timing of gluten-introduction into the infant diet was associated with the appearance of CD autoimmunity, whereas no effect of the duration of breast-feeding was observed (Norris *et al.*, 2005). The authors speculate that the effect of breast-feeding is not seen in North-American infants since gluten is introduced in the form of infant cereals, which are not replacements for breast milk and their use does not correlate with breast-feeding duration.

It is extremely difficult to dissect the effect of different mediators in the pathogenic process leading to the manifestation of T1D or appearance of surrogate markers such as beta-cell autoantibodies. The intake of dietary items introduced at the weaning, such as cow milk formula, is lower if the breast-feeding is still continued. The infants with a short duration of total breast-feeding (i.e. breast-fed less than 4 months) have been exposed to higher doses of cow milk formula at the age of 3 months than those infants who have been reported to have duration of exclusive breast-feeding less than 4 months. This kind of dose-effect was seen in a previous study of primary immunization to dietary insulin during infancy: the highest antibody levels to dietary insulin were seen in the infants who did not receive breast milk at the age of 3 months, whereas the levels were lower in those infants who were breast-fed during the first exposure to cow milk formula (Vaarala *et al.*, 2002). Also in the studies of risk of CD, children with CD were exposed to a larger amount of gluten at first exposure than children without CD (Ivarsson *et al.*, 2002). Furthermore, the immunization to dietary bovine insulin is modified by human insulin in breast-milk so that exposure to high levels of breast-milk insulin is associated with decreased levels of antibodies binding to dietary insulin (Tiittanen *et al.*, 2005). Accordingly, human insulin in breast-milk may attenuate the immunization to insulin by the mechanisms of oral tolerance

(Harrison & Honeyman, 1999). We conclude that it is important to breastfeed while introducing new food items during the weaning time.

In previous studies of children with increased genetic risk of T1D (Norris *et al.*, 2003) or a first degree relative with T1D (Couper *et al.*, 1999; Ziegler *et al.*, 2003) no association between duration of either total or exclusive breast-feeding and beta-cell autoimmunity was found. Also, early introduction of food supplement containing cow milk did not increase the risk of beta-cell autoimmunity (Norris *et al.*, 2003; Ziegler *et al.*, 2003). The prospective studies in Germany (Ziegler *et al.*, 2003) and USA (Norris *et al.*, 2003) did not reveal any association between duration of breast-feeding and beta-cell autoimmunity, as was found in the Finnish study (T Kimpimäki *et al.*, 2001) as well as in ours. The different findings in these studies may be due to differences in the populations or reflect different infant feeding practices in different countries.

One effect of early discontinuation of breast-feeding and subsequent introduction of formula may be an increased weight gain, a risk factor for T1D (Johansson *et al.*, 1994; Hyppönen *et al.*, 1999) which results in impaired insulin sensitivity causing elevated blood glucose (the accelerator-hypothesis) (Wilkin, 2001). When weaning to formula in Germany and USA, a hydrolysed formula is often used (Åkerblom *et al.*, 2005), which is less immunogenic (Vaarala *et al.*, 1995). These kinds of formulas are less often used in Sweden (according to the recommendations of the National Board of Health and Welfare) and in Finland (Åkerblom *et al.*, 2005). The high frequency of children receiving breast-milk at 3 months of age in our study-cohort correlates with numbers from the entire ABIS study population (Brekke *et al.*, 2005) as well as the National Board of Health and Welfare (Official Statistics of Sweden, 2000).

We do not expect a large number of children with T1D in our study population at this age and the end-point is therefore beta-cell autoimmunity. One may speculate if the beta-cell autoimmunity is transient or if the aetiology differs from that of T1D. It is possible that other environmental factors trigger the initial beta-cell autoimmunity than those factors which later cause the following T1D (Knip *et al.*, 2005). Despite this, the environmental factors which trigger the beta-cell autoimmunity determine the population in risk of T1D. Additional studies may shed light on the importance of these factors, such as short duration of breast-feeding, in the process from beta-cell autoimmunity to clinical disease.

Paper IV: Risk genes & autoantibodies in children from the general population

Beta-cell autoantibodies are mostly studied in either first degree relatives of probands with T1D or in children with increased genetic risk of T1D. The present knowledge on the development of beta-cell autoimmunity in these populations may not be relevant for the general population. Our aim was therefore to retrieve information on the initiation of beta-cell autoimmunity in 5-6 year old children from the general population and also on the association with HLA risk haplotypes and insulin gene VNTR polymorphism. The cut off levels for autoantibody positivity used in this study was the 98th or 99th percentile.

Frequencies of HLA haplotypes and *INS*-23 genotypes

In Swedish children from the general population the two most common haplotypes associated with susceptibility were DRB1*0401/2/4/5-DQA1*0301-DQB1*0302 (DR4-DQ8) and (DR3)-DQA1*05-DQB1*02 (DR3-DQ2) The three most common haplotypes associated with protection was (DR15)-DQB1*0602, (DR5)-DQA1*05-DQB1*0301 and (DR1301)-DQB1*0603 (Table 25).

Table 25: Frequencies of the five most commonly detected haplotypes associated with risk or protection among non-diabetic Swedish children.

	Haplotype	N (%)
Risk	(DR3)-DQA1*05-DQB1*02	163/711 (22.9)
	DRB1*0401/2/4/5-DQA1-0301-DQB1*0302	212/711 (29.8)
Protection	(DR15)-DQB1*0602	203/711 (28.6)
	(DR5)-DQA1*05-DQB1*0301	129/711 (18.1)
	(DR1301)-DQB1*0603	75/711 (10.5)

The insulin gene polymorphism was analysed as -23 A/T polymorphism. Among non-diabetic Swedish children the frequency of the AA genotype was 306/643 (47.6%), of the A/T genotype 278/643 (43.2%), of the T/T genotype 59/634 (9.2%) and 337/643 (52.4%) carried either A/T or T/T genotype (non-A/A genotype).

Beta-cell autoantibodies in relation to HLA haplotypes or genotypes

The number of children positive for GADA above the 99th percentile was higher in children with the DR4-DQ8 (10/182, 5.5%) compared to children without this

haplotype (6/459, 1.3%) ($p=0.005$; Table 26). Similar results were found for GADA above the 98th but not for IAA or IA-2A or any combination of autoantibodies (Table 26). For DR3-DQ2, no differences were found (Table 27).

Table 26: Frequencies of autoantibodies above the 98th or 99th percentile in non-diabetic Swedish children with or without the DR4-DQ8 haplotype. Frequencies were compared by Chi-square test.

Autoantibody	DR4-DQ8		Total N	p value
	No N (%)	Yes N (%)		
GADA, 98th	14/459 (3.1)	13/182 (7.1)	641	0.020
GADA, 99th	6/459 (1.3)	10/182 (5.5)	641	0.005
IAA, 98th	26/329 (7.9)	11/132 (8.3)	461	0.9
IAA, 99th	11/329 (3.3)	5/132 (3.8)	461	1.0
IA-2A, 99th	4/469 (0.9)	5/193 (2.6)	662	0.2
GADA and/or IAA, 98th	40/487 (8.2)	22/202 (10.9)	689	0.3
GADA, IAA and/or IA-2A, 99th	21/499 (4.2)	15/212 (7.1)	711	0.1

Table 27: Frequencies of autoantibodies above the 98th or 99th percentile in non-diabetic Swedish children with or without the DR3-DQ2 haplotype. Frequencies were compared by Chi-square test.

Autoantibody	DR3-DQ2		Total N	p value
	No N (%)	Yes N (%)		
GADA, 98th	20/488 (4.1)	7/153 (4.6)	641	0.8
GADA, 99th	13/488 (2.7)	3/153 (2.0)	641	0.9
IAA, 98th	29/353 (8.2)	8/108 (7.4)	461	0.8
IAA, 99th	13/353 (3.7)	3/108 (2.8)	461	0.9
IA-2A, 99th	8/505 (1.6)	1/157 (0.6)	662	0.6
GADA and/or IAA, 98th	47/529 (8.9)	15/160 (9.4)	689	0.8
GADA, IAA and/or IA-2A, 99th	30/548 (5.5)	6/163 (3.7)	711	0.4

In children with the protective haplotype (DR15)-DQB1*0602, the prevalence of autoantibody positive children was lower. The prevalence of children positive for GADA and/or IAA above the 98th percentile and/or IA-2A above the 99th percentile was lower in children with (DR15)-DQB1*0602 (12/203, 5.9%) compared to children without this haplotype (56/508, 11.0%) ($p=0.036$; Table 28). Similarly, the prevalence of children positive for GADA and/or IAA above the 98th percentile tended to be lower in children with (DR15)-DQB1*0602 (Table 28). No differences in frequency of autoantibodies were found with the protective haplotypes (DR5)-DQA1*05-DQB1*0301 and (DR1301)-DQB1*0603 (data not shown). The number of children positive for GADA, IAA or IA-2A, or combinations of these, was not affected by other HLA haplotypes associated with susceptibility or protection (data not shown).

Table 28: Frequencies of autoantibodies above the 98th or 99th percentile in non-diabetic Swedish children with or without the protective (DR15)-DQB1*0602 haplotype. Frequencies were compared by Chi-square test.

	(DR15)-DQB1*0602		Total N	p value
	No	Yes		
	N (%)	N (%)		
GADA and/or IAA, 98th	51/495 (10.3)	11/194 (5.7)	689	0.056
GADA and/or IAA, 98th and/or IA-2A, 99th	56/508 (11.0)	12/203 (5.9)	711	0.036
GADA, IAA and/or IA-2A, 99th	30/508 (5.9)	6/203 (3.0)	711	0.1

Table 29: Frequencies of IAA or GADA above the 98th or 99th percentile as single autoantibodies or in combination in non-diabetic children with or without HLA risk genotypes (see Table 30 for definitions). Frequencies were compared by Chi-square test.

	HLA genotype			Total N	p value
	High risk	Risk	No risk		
	N (%)	N (%)	N (%)		
GADA, 98th	4 (6.8)	12 (4.8)	10 (3.0)	640	0.3
IAA, 98th	2 (4.9)	16 (9.0)	19 (7.9)	460	0.7
GADA and/or IAA, 98th	6 (9.5)	26 (9.6)	29 (8.2)	688	0.8
GADA, IAA and/or IA-2A, 99th	4 (6.0)	13 (4.7)	18 (4.9)	710	0.9
GADA, IAA, 98th and/or IA-2A, 99th	6 (9.1)	28 (10.1)	33 (9.0)	709	0.9

The frequencies of GADA or IAA as single autoantibodies or in combination were similar in children with or without HLA risk genotypes defined as “High risk”, “No risk” or “Risk” (Table 29). The definition of the HLA genotypes defined as “High risk”, “No risk” or “Risk” are given in Table 30.

Table 30: Definitions of the HLA "high risk" and "no risk" genotypes used. The "No risk" haplotypes are associated with protection (upper part of table) or are neutral (lower part of table). "Risk" is defined as having one of the high risk haplotypes in combination with one of the haplotypes associated with no risk.

HLA genotypes	HLA genotypes
High risk	No risk
two of these haplotypes	two haplotypes: Protective or Neutral
DRB1*0401/2/4/5-DQA1*0301-DQB1*0302	(DR15)-DQB1*0602
(DR3)-DQA1*05-DQB1*02	(DR5)-DQA1*05-DQB1*0301
	(DR7)-DQA1*0201-DQB1*0303
	(DR14)-DQB1*0503
	DRB1*0403-DQA1*0301-DQB1*0302
	(DR1301)-DQB1*0603
	DQA1*0301-DQB1*0301
	(DR7)-DQA1*0201-DQB1*02
	(DR1302)-DQB1*0604
	(DR9)-DQA1*03-DQB1*0303
	(DR8)-DQB1*04
	(DR1)-DQB1*0501
	(DR2)-DQB1*0601
	(DR16)-DQB1*0502
	DQB1*0609

Beta-cell autoantibodies in relation to polymorphism in the insulin gene

The frequency of IAA positive children was similar in children with *INS*-23 A/A, A/T or T/T genotypes even when children with A/T and T/T were grouped together (Table 31). The levels of IAA were similar in children with A/A, A/T or T/T genotype, also in children with HLA risk haplotype (Table 32). No differences in frequencies of GADA or IA-2A positivity, or any combination of autoantibodies, were found in children with different *INS*-23 genotypes (data not shown).

Table 31: Frequencies of IAA above the 98th or 99th percentile in non-diabetic children in relation to INS A/A, A/T or T/T genotypes, or to A/A and non-A/A (children with A/T or T/T genotype). Frequencies were compared by Chi-square test.

INS genotype	IAA, 98th perc.	IAA, 99th perc.
	N (%)	N (%)
A/A	15/202 (7.4)	8/202 (4.0)
A/T	20/182 (11.0)	8/182 (4.4)
T/T	4/42 (9.5)	2/40 (4.4)
Total N	426	426
p value	0.5	1.0
A/A	15/202 (7.4)	8/202 (4.0)
non-AA	24/224 (10.7)	10/224 (4.5)
Total N	426	426
p value	0.2	0.8

Table 32: Frequencies of IAA above the 98th or 99th percentile in non-diabetic children in relation to INS A/A, A/T or T/T genotypes, or to A/A and non-A/A (children with A/T or T/T genotype), in children matched for A) HLA DR4-DQ8 or B) DR3-DQ2 haplotype. Frequencies were compared by Chi-square test.

A

Autoantibody	N (%)	DR4-DQ8			p value	Total N
		INS genotype				
		A/A	A/T	T/T		
IAA, 98th perc.	3/37 (8.1)	5/23 (21.7)	1/5 (20.0)	none	65	
IAA, 99th perc.	1/37 (2.7)	3/23 (13.0)	0/5	none	65	
		A/A	non-AA	p value		
		3/37 (8.1)	6/28 (21.4)	0.2		
		1/37 (2.7)	3/28 (10.7)	none		

Continues on next page

Table 32: Continued.

B

Autoantibody		DR3-DQ2 INS genotype			p value	Total N
		A/A	A/T	T/T		
IAA, 98th perc.	N (%)	3/37 (8.1)	5/23 (21.7)	1/5 (20.0)	none	65
IAA, 99th perc.	N (%)	1/37 (2.7)	3/23 (13.0)	0/5	none	65
		A/A	non-AA		p value	
		3/37 (8.1)	6/28 (21.4)		0.2	
		1/37 (2.7)	3/28 (10.7)		none	

none; no p value because expected count < 5 in 20% of the cells and minimum expected count < 1

Discussion

We found that Swedish children from the general population with the risk associated haplotype DR4-DQ8 had an increased frequency of GADA at 5-6 years of age. In previous studies, GADA has been associated with DQ2 in subjects with T1D (Sabbah *et al.*, 1999; Graham *et al.*, 2002) and in genetically predisposed Finnish children (Hermann *et al.*, 2005). In German schoolchildren with autoantibodies, the frequency of GADA was increased in children with haplotypes conferring either risk or protection of T1D (Schlosser *et al.*, 2003). The frequencies of the risk associated haplotypes DR4-DQ8 and DR3-DQ2 found in our population of non-diabetic Swedish children corresponds to earlier studies in Swedish children (Kockum *et al.*, 1999). These haplotypes have previously been reported to be associated with T1D in the Swedish populations (Sanjeevi *et al.*, 1995; Sanjeevi *et al.*, 1996).

We found that 5-6 year old non-diabetic Swedish children with the protective haplotype (DR15)-DQB1*0602 had a decreased frequency of GADA, IAA and/or IA-2A, indicating that a haplotype commonly associated with decreased risk of T1D also is associated with decreased risk of developing beta-cell autoimmunity at a population level. The frequencies of autoantibodies were not however affected by the protective haplotypes (DR5)-DQA1*05-DQB1*0301 and (DR1301)-DQB1*0603.

The frequency of the *INS*-23 A/A genotype, associated with increased risk of T1D, found in our population of non-diabetic Swedish children is similar to frequencies in

healthy subjects in both Sweden (Graham *et al.*, 2002) and Western Europe (van der Auwera *et al.*, 1995; Walter *et al.*, 2003). We found that the frequency of IAA positive children, as well as GADA or IA-2A positive children, was similar for non-diabetic 5-6 year old children with *INS*-23 A/A, A/T or T/T genotype. An association between the risk *INS* genotype and frequency of IAA has been reported previously in genetically predisposed Finnish children who also were positive for islet cell antibodies (ICA) indicating perhaps stronger induction of diabetes associated autoimmunity (Hermann *et al.*, 2005). Walter and co-authors found an association between autoantibody positivity and *INS* risk genotype in children of parents with T1D (Walter *et al.*, 2003). The children in this study (the ABIS study) have not been selected by means of increased risk of T1D by heredity or genetic risk factors. This suggests that emergence of IAA or other autoantibodies are not modified by *INS* polymorphism if other genetic risk factors are absent. The emergence of beta-cell autoimmunity in our study population is associated with the HLA haplotype conferring high risk of T1D (i.e. HLA DR4-DQ8), but the impact of other T1D associated risk genotypes or haplotypes in the development of autoantibodies seems to be small in a general population.

These results suggest that the risk of beta-cell autoimmunity in the general population is not strongly associated with other risk genes of T1D than the HLA DQB1*0302-DRB1*0401/2/4/5 haplotype, indicating that environmental factors are more important determinants in the induction of beta-cell autoimmunity.

The risk genes of T1D may be more important for the progression from beta-cell autoimmunity to the clinical disease, although progression to T1D in Finnish schoolchildren with multiple autoantibodies but without the risk associated allele DQB1*0302 has been reported (Kulmala *et al.*, 2001). However, the Finnish schoolchildren were a little older than the children in our study, and the HLA genotypes associated with increased risk of T1D is most frequently found in young patients (Karjalainen *et al.*, 1989; Komulainen *et al.*, 1997).

When the appearance of beta-cell autoimmunity is screened in a population with hereditary or genetic risk of T1D, the frequency of beta-cell autoimmunity is higher when compared to the general population (Barker *et al.*, 2004; Hummel *et al.*, 2004; Franke *et al.*, 2005; Kukko *et al.*, 2005). It is therefore regarded as more efficient to study a group of individuals at increased risk of T1D in order to design a strategy for prediction of T1D that later could be applied to the general population. It may be that screening of autoantibodies in the general population without pre-screening of HLA risk genes is less effective in the prediction of T1D since fewer children will develop T1D, however it may reveal the environmental triggers of beta-cell autoimmunity. These triggering factors seem to work also in the general population and their effect is not restricted to children with genetic risk of T1D. The interaction of these environmental triggers with HLA is important. In view of our findings, as well as

others (Karjalainen *et al.*, 1989; Komulainen *et al.*, 1997), the presence of HLA risk alleles is not a prerequisite for beta-cell autoimmunity or T1D.

SUMMARY & CONCLUSIONS

We have studied the development of beta-cell autoantibodies in children from the general population and in non-diabetic children with an allergic heredity as well as in patients with newly diagnosed T1D. The children from the general population participated in the ABIS study and represent the cohort of children born in south-east Sweden between October 1997 and October 1999. They were not selected by hereditary risk or increased genetic risk of T1D, and in this aspect, the design of the ABIS study was unique.

In children from the general population, we that found an association between positivity for GADA and/or IAA at the age of 5-6 years and a short duration of total breast-feeding. We also found an association between positivity for GADA, IA-2A and IAA and short duration of exclusive breast-feeding. Our findings suggest that breast-feeding has a long term protective effect on the risk of beta-cell autoimmunity in children from the general population that is seen several years after completing breast-feeding. It is especially interesting that a short duration of total-breastfeeding was associated with positivity for IAA. It has been hypothesised that immunisation to bovine insulin, present in the formula used at weaning, could explain the link between early introduction of cow milk and increased risk of T1D. Although retrospective studies have reported an association between a short duration of total or exclusive breastfeeding and risk of T1D, several prospective studies have failed to do so in relation to beta-cell autoimmunity. One could hypothesise that the lack of association could be due to differences in the populations studied or weaning to different kind of formulas compared to the Swedish children studied here. Findings of associations of breast-feeding and T1D as well as beta-cell autoantibodies have been reported from Finland, a country with high incidence of T1D, but no association was reported in Germany or USA, countries with lower incidence of T1D. It can not be ruled out that these differences could be explained by differences in the populations. Early discontinuation of breast-feeding may coincide with other environmental and genetic factors resulting in a higher impact of the short duration of breast-feeding on the risk of beta-cell autoimmunity in for example Swedish and Finnish children.

We also found that children from the general population who carried the DR4-DQ8 haplotype more often were positive for GADA at 5-6 years of age than children without this haplotype. We found no association of GADA with DR3-DQ2 haplotype or between these two haplotypes and any of the other autoantibodies, single or in combinations. We did not find any association between IAA and polymorphism in the insulin gene among children from the general population, and perhaps this association may only exist in children who are progressing to T1D, i.e. with positivity for more than one autoantibody. The results suggest that beta-cell autoimmunity in children from the general population is not strongly associated with any risk genes of T1D other than DR4-DQ8. The risk genes are probably more important in defining the risk

of T1D, i.e. the development from beta-cell autoimmunity to T1D indicating that environmental factors are important determinants in the induction of beta-cell autoimmunity.

The effect of HLA risk genes on development of beta-cell autoantibodies in the general population is less known, although Finnish schoolchildren with multiple autoantibodies, progressed to T1D without carrying the risk associated allele DQB1*0302. When the appearance of beta-cell autoimmunity is screened in a population with hereditary or genetic risk of T1D, the frequency of beta-cell autoimmunity is higher when compared to the general population. The study of individuals at increased risk of T1D is therefore regarded as more efficient in order to design a strategy for prediction of T1D. It may be that screening of autoantibodies in the general population without pre-screening of HLA risk genes or by selecting first degree relatives is less effective in the prediction of T1D, since fewer children will develop T1D, however it may reveal the environmental triggers of beta-cell autoimmunity. These triggering factors seem to affect also the children in the general population and not only the children predisposed to T1D. The interaction of these environmental triggers with HLA is important. In view of our findings, as well as others (Karjalainen et al., 1989; Komulainen et al., 1997), the presence of HLA risk alleles is not a prerequisite for beta-cell autoimmunity or T1D.

In the non-diabetic children with an allergic heredity we found that several children have an immune response to beta-cell autoantigens, but the autoantibodies are found at low levels. Generally, cut off limits based on for example the 95th percentile of healthy children is used to define autoantibody positivity and negativity in the study population. We decided to use a different approach and study the immune response to beta-cell autoantigens in children without T1D by analysing all detectable autoantibodies. We found that GADA was detectable in almost all children, IA-2A in about half and IAA in about 10% of the children. Also, the levels of these autoantibodies fluctuated with age and different patterns of fluctuations were seen for GADA and IA-2A, which may reflect differences in the immune response to the autoantigens.

In patients with newly diagnosed T1D, we found some differences between patients from a high incidence country (Sweden) and a country with substantially lower incidence (Lithuania). Among the Swedish patients, the prevalence of IAA and GADA was higher than in Lithuanian patients. Also, the prevalence of multiple autoantibodies was higher in Swedish patients compared to Lithuanians. The risk genes DR4-DQ8 and the heterozygous high risk combination DR4-DQ8 with DR3-DQ2 was more common among the Swedish patients than Lithuanians patients. When matched according to HLA DR4-DQ8 or DR3-DQ2 genotype, the differences between Swedish and Lithuanian patients in the prevalence of GADA remained and when matched for the high risk genotypes of the insulin gene polymorphism, the differences in the

prevalence of IAA remained. Our findings indicate that an autoimmune response to insulin and GAD₆₅ is more pronounced in a high incidence area compared to a low incidence area. We also found associations between beta-cell autoantibodies and clinical characteristics at diagnosis. Patients with low levels of IAA had a higher level of HbA1c and ketones, indicating patients without IAA or with low levels of IAA have a more severe onset of T1D.

The differences in clinical parameters between Swedish and Lithuanian patients may be due to an earlier diagnosis and hence less severe onset in Sweden. However, the differences in frequency of risk genes and beta-cell autoantibodies may be caused by the differences between the populations in these two areas. A different genetic background and different environmental triggers during infancy and childhood may precipitate beta-cell autoimmunity and T1D more often in Swedish children than in Lithuanian children.

From the findings of our studies I conclude that the aetiology of T1D may be different in different populations due to differences in phenotype. The triggering factors of beta-cell autoimmunity may also be different or exert different effects in high incidence areas as compared to low incidence areas. It is already extremely difficult to dissect the effect of different mediators in the pathogenesis of T1D e.g. the interaction of different environmental factors together with the genetic risk of T1D. It may, however, be even more complicated if there are also differences between populations that need to be taken into account. This is speculative, and the results of ongoing multi-national studies like the TRIGR study and the TEDDY study will be very interesting.

In conclusion, beta-cell autoantibodies are detectable in children from the general population and in non-diabetic children with allergic heredity. The levels of autoantibodies below the generally accepted cut off levels are seen in many non-diabetic children. In children from the general population, only positivity to GADA at 5-6 years of age seems to have any association with T1D related genetic risk. Short duration of total or exclusive breast-feeding is a risk factor for beta-cell autoantibodies at the age of 5-6 years in children from the general population. Children with newly diagnosed T1D in a high incidence area carry risk genes and have autoantibodies more often than newly diagnosed children from an area with lower incidence, indicating perhaps different disease phenotypes.

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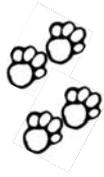
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