ASSESSMENT OF RISK FOR TYPE 1 DIABETES IN CHILDREN OF AFFECTED FAMILIES AND IN THE GENERAL POPULATION: ROLE OF IMMUNOLOGICAL AND METABOLIC MARKERS

by

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Dedicated to those who do not despise the day of minor beginnings

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ABSTRACT

Background

Type 1 diabetes (T1D) is an autoimmune disease in which the insulin producing beta cells of pancreatic islets are gradually destroyed. The clinical presentation of T1D is preceded by a prodromal phase characterized by the appearance of diabetes-associated autoantibodies in the circulation. Both the timing of the appearance of autoantibodies and their quality have been used in the prediction of T1D among first-degree relatives (FDR) of diabetic patients. So far, no generally accepted strategies for systematically identifying individuals at increased disease risk in the general population have been established, although the majority of new cases originate in this part of the population. Since the incidence of T1D continues to increase throughout the world and the burden it causes is significant, efforts aimed at delaying or preventing the clinical onset of T1D are important. Reliable disease prediction is a corner stone both for successful intervention trials and for effective and safe preventive therapies in the future.

Aims

This thesis work aimed at assessing the predictive role of diabetes-associated immunologic and metabolic risk factors in the general population, and defining risk profiles of these factors in comparison with data obtained from studies on family members of affected patients.

Subjects and Methods

Observations of the current studies are reported in original publications I-IV. Study subjects in publication I were derived from the Childhood Diabetes in Finland Study (DiMe; n=755) and the Cardiovascular Risk in Young Finns Study (LASERI; n=3475). The DiMe cohort comprised siblings of children with newly diagnosed T1D, and the LASERI cohort randomly selected healthy Finnish children, representing populations of the five University Hospital Districts of Finland. Blood samples for autoantibody assays were obtained from subjects of both cohorts at the mean age of 10.0 and 10.8 years, respectively, after which the participants were observed for progression to clinical T1D for a median of 15 years. Predictive role of autoantibodies against glutamic acid decarboxylase (GADA) and islet antigen 2 (IA-2A) were assessed and compared between the two cohorts.

Study subjects (n=7410) for publication II were derived from the ongoing Finnish Type 1 Diabetes Prediction and Prevention Study (DIPP) in which babies born in Turku, Oulu and Tampere University Hospitals are screened for HLA-conferred risk for T1D. Infants with diabetes-prone HLA genotypes are enrolled to follow-up study in which various diabetes-related risk factors are recorded and samples for autoantibody assays obtained on a regular basis.

The role of islet cell antibodies (ICA) alone and in combinations with GADA, IA-2A, and autoantibodies against insulin (IAA), were assessed in children participating in the DIPP study (“DIPP children”) and the results obtained were compared with those

reported from studies on family members of patients with T1D. For publication III, 64 IAA-positive DIPP children who progressed to overt T1D were matched with 64 healthy IAA-positive control children to assess the predictive value of IAA affinity.

DIPP children with persistent positivity for at least two of the diabetes-associated autoantibodies were enrolled to a randomized, double-blinded, placebo-controlled intervention trial with nasally administrated insulin after reaching the age of 1 year. Before starting the intervention treatment, participants underwent oral and intravenous glucose tolerance tests (OGTT and IVGTT, respectively). Focus in publication IV was on the predictive value of metabolic markers in persistently multipositive DIPP children (n=218) with results from at least one OGTT and one IVGTT.

Results

By combining HLA and autoantibody screening, T1D risks that are similar to those reported for autoantibody-positive FDRs of affected children can be observed in children and adolescents representing the general population. Predictive sensitivity of GADA, IA-2A, and their combination is of same magnitude both in siblings of affected children and in the general population (68% vs. 50% for GADA; 58% vs. 43% for IA-2A; 48% vs. 36% for combined positivity, respectively, $P>0.05$ for all comparisons). Cumulative disease risks of single GADA and IA-2A positivity were higher in siblings of affected children than in the general population-based cohort (61% vs. 24%, respectively, $P<0.001$ for GADA; 74%; vs. 32%, respectively, $P=0.002$ for IA-2A), but combined positivity indicated similar cumulative risk in both populations (83% vs. 86%, respectively, $P=0.89$).

Natural progression rate to clinical T1D is high in genetically susceptible children testing persistently positive for multiple autoantibodies, including persistent positivity for IAA. Measurement of IAA affinity failed in stratifying the disease risk assessment in young IAA-positive children with HLA-conferred disease susceptibility. The affinity of IAA did not increase during the prediabetic period in the current study subjects.

Young age at seroconversion, increased weight-for-height, decreased early insulin response, and increased IAA and IA-2A levels predict T1D in young children with HLA-conferred disease susceptibility and signs of advanced beta-cell autoimmunity. Insulin resistance has a minor impact on progression to T1D after the initiation of the disease process in young normal-weight children with HLA-conferred disease susceptibility.

Conclusions

Combined genetic HLA-based screening and regular autoantibody measurements reveal similar disease risks in children of the general population as those seen in autoantibody-positive siblings of children with T1D. Our observations confirmed that the assessment of disease risk can be stratified further by studying glucose metabolism of prediabetic subjects, and that these prediction strategies result in profiles with highly variable disease risks. As these screening efforts are feasible also in practice, the information now obtained can be exploited when designing intervention trials aimed at secondary prevention of T1D, and for the identification of individuals at increased risk for T1D, as soon as the first effective preventive measures for T1D have been established.

ABBREVIATIONS AND DEFINITIONS

APC	Antigen presenting cell
CD	Cluster of differentation
CI95%	95% confidence intervals
CTL	Cytotoxic T-lymphocytes
CTLA4	Cytotoxic T-lymphocyte-associated antigen 4
DAA	Diabetes-associated autoantibodies
DAISY	Diabetes Autoimmunity Study in the Young
DASP	Diabetes Autoantibodies Standardization Program
DIABIMMUNE	Project testing the hygiene hypothesis in pathogenesis of type 1 diabetes
DiMe	Childhood Diabetes in Finland Study
DIPP	Finnish Type 1 Diabetes Prediction and Prevention Study
DPT-1	Diabetes Prevention Trial -Type 1
ENDIT	European Nicotinamide Diabetes Intervention Trial
FDR	First-degree relative
FPIR	First-phase insulin release
GAD	Glutamic acid decarboxylase
GADA	Autoantibodies against the 65 kD isoform of glutamic acid decarboxylase
GDM	Gestational diabetes mellitus
HLA	Human leukocyte antigen
HOMA-IR	Homeostasis model assessment for insulin resistance
HOMA-IR/FPIR	Relative insulin resistance
HR	Hazard ratio
IAA	Insulin autoantibodies
IA-2	Islet antigen 2
IA-2A	Autoantibodies against islet antigen 2
IC50	Half maximal inhibitory concentration
ICA	Islet cell antibodies
IDDM	Insulin-dependent diabetes mellitus
Ig	Immunoglobulin

INS	Gene coding for insulin
IVGTT	Intravenous glucose tolerance test
JDFU	Juvenile Diabetes Research Foundation Unit
LADA	Latent autoimmune diabetes mellitus in adults
LASERI	Study on Cardiovascular Risk Factors in Young Finns
LR-	Likelihood ratio after negative result
LR+	Likelihood ratio after positive result
MODY	Maturity onset diabetes of the young
Non-progressors	Study subjects who did not develop clinical T1D during follow-up
NPV	Negative predictive value
OGTT	Oral glucose tolerance test
OR	Odds ratio
PBMC	Peripheral blood mononuclear cells
Persistent multipositivity	Positivity for ≥2 diabetes-related autoantibodies in ≥2 consecutive blood samples taken ≥3 months apart
PND	Persistent neonatal diabetes
PPV	Positive predictive values
Prefix “p”	Persistent autoantibody positivity
Progressors	Study subjects who developed clinical T1D during follow-up
PTPN22	Lymphoid-specific protein tyrosine phosphatase-like protein 22
PTPs	Protein tyrosine phosphatases
QUICKI	Quantitative Insulin-Sensitivity Check Index
r_S	Spearman’s correlation coefficient (Rho)
RU	Relative Units
SLC30A8	Gene coding for zinc transporter 8 (ZnT8)
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
SPSS	Statistical Package for the Social Sciences
T1D	Type 1 diabetes
TND	Transient neonatal diabetes
TRIGR	Trial to Reduce IDDM in the Genetically at Risk
WHO	World Health Organization

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that have been reprinted with permission of the copyright holders:

Publication I: Siljander H, Veijola R, Reunanen A, Virtanen SM, Åkerblom HK, Knip M. Prediction of type 1 diabetes among siblings of affected children and in general population. Diabetologia 50:2272-2275, 2007

Publication II: Siljander H, Simell S, Hekkala A, Lähde J, Simell T, Vähäsalo P, Veijola R, Ilonen J, Simell O, Knip M. Predictive characteristics of diabetes-associated autoantibodies among children with HLA-conferred disease susceptibility in the general population. Diabetes 58:2835-2842, 2009

Publication III: Siljander H, Härkönen T, Hermann R, Simell S, Hekkala A, Salonsaari R-T, Simell T, Simell O, Ilonen J, Veijola R, Knip M. Role of insulin autoantibody affinity as a predictive marker for type 1 diabetes in young children with HLA-conferred disease susceptibility. Diabetes Metab Res Rev 25:615-622, 2009

Publication IV: Siljander H, Hermann R, Hekkala A, Lähde J, Keskinen P, Ilonen J, Simell O, Veijola R, Knip M. Insulin secretion and sensitivity in the prediction of type 1 diabetes among children with advanced beta-cell autoimmunity. Submitted for publication.

INTRODUCTION

Type 1 diabetes (T1D) is caused by immune-mediated destruction of pancreatic beta cells that is preceded by a preclinical phase during which diabetes-associated autoantibodies appear in the circulation. The timing of appearance and the identity of autoantibodies against beta cells have been used in the prediction of T1D among first-degree relatives of diabetic patients, but so far no consensus has been reached as to strategies aimed at identifying individuals at increased disease risk in the general population, although around 90% of new cases with T1D are derived from that population. The work presented in this thesis sets out to provide new information on prediction of T1D in the pediatric general population for filling some of the gaps in our current knowledge.

REVIEW OF LITERATURE

CLASSIFICATION OF DIABETES

Throughout centuries, diabetes was recognized as a deadly ailment in which the patient's flesh and limbs melt into urine that has a sweet taste (Brill 2008), and during the pre-insulin era the life expectancy of the diabetic patient was short and mortality associated with the disease high. During the 19th century several basic concepts of the modern medical knowledge became established and the nature of the endocrine and exocrine organs characterized. In 1869 a German medical student Paul Langerhans observed that the pancreas contains two types of cells: those secreting "the normal pancreatic juice" and nests of cells of unknown function. The latter were named later the "islets of Langerhans" (Rennie and Fraser 1907), and already in 1908, the first experiments were performed to treat glucosuria with injections of pancreatic extracts. Even if these extracts were only partially purified and caused severe side effects, in 1922 these experiments lead to the identification of insulin by Frederic Banting and his colleagues (Banting et al. 1922). Today diabetes is considered as a serious but treatable condition in which the diabetic individuals either produce insufficient amounts of insulin or the effect of secreted insulin is suboptimal due to insulin resistance or defects in the insulin signaling system in the target cells.

After the establishment of insulin therapy, the heterogenic nature of diabetes became evident. In the 1950s diabetes was reported to manifest in two major types that differed from each other by the need of exogenous insulin: the insulin-dependent diabetes (type 1 diabetes, T1D) and the non-insulin-dependent diabetes (type 2 diabetes, T2D) (Bornstein and Lawrence 1951). Two decades later, in the 1970s, the association of T1D and autoimmunity was discovered and the first diabetes-related autoantibodies, islet cell antibodies (ICA), were characterized (Bottazzo et al. 1974, MacCuish et al. 1974). The modern concept of diabetes includes also gestational diabetes mellitus (GDM), latent autoimmune diabetes of the adulthood (LADA), maturity onset diabetes of the young (MODY), transient or persistent neonatal diabetes (TND and PND, respectively), and double diabetes or "1.5 diabetes", which refers to diabetes with clinical characteristics of both autoimmune diabetes and insulin resistance-related T2D (World Health Organization 1999, Gillam et al. 2005).

In addition to the subtypes mentioned above, diabetes is observed in certain diseases and polyendocrinopathies, such as Wolfram and Alström syndromes, Immunodysregulation Polyendocrinopathy Enteropathy X-linked syndrome (IPEX), and Autoimmune Polyendocrinopathy-Candidiasis-Ectodermal Dystrophy (APECED). In these syndromes, diabetes appears usually during early infancy, except for APECED, where diabetes is often diagnosed at adult age, and even though these conditions are rare, together they account for up to 5% of cases of diabetes among children (Barrett 2007). From the scientific point of view these mutation-based conditions are significant, since they provide possibilities to study the basic mechanisms of insulin and glucose metabolism and to correlate the clinical diabetes phenotype with its genotype. The deeper understanding of the gene

and protein interactions in the pathogenesis of diabetes may enable us to develop new therapeutic measures for diabetes in the future.

THE IMMUNE SYSTEM

BARRIER FUNCTION

The first line of defense against intruders into the body is the mechanical, physical, and chemical barrier provided by the skin and the mucous membranes. The exposed surfaces of the body become colonized soon after birth by protective microbes that participate in the immunologic defense by competing with pathogens for space and nutrients, and by driving the immune responses of the host towards normal immunotolerance. The neonatal period is a critical time in life in terms of the induction of oral tolerance (Faria 2005). The postnatal maturation of the intestine and the development of the normal mucosal barrier function require the establishment of the normal microbial flora and a well-timed and dosed stimulus by nutritional antigens (Vaarala 1999, Hänninen 2000). Breastfeeding has been shown to enhance the development of immunotolerance, whereas certain enteral infections, especially those caused by enteroviruses or rotaviruses, have been hypothesized to disturb the mucosal barrier function (Honeyman et al. 2000, Salminen et al. 2003). The increased permeability and the transfer of foreign antigens through the gut mucosa could in turn lead to sensitization and/or false recognition of nutrition-originating antigens.

INNATE AND ADAPTIVE IMMUNITY

The main challenge of the immune system is to segregate foreign structures from normal self-originating ones, and consequently, development and maintenance of this self-tolerance is crucial for the efficacy of the immune system. Immunologic responses can be divided into innate and adaptive immunity, reflecting the level of complexity and the evolution of the immune processes required, or into humoral and cell-mediated immunity, indicating the molecular mechanisms by which the immune responses are mediated. Innate immune responses are most often simple, efficacious, and of maximal intensity, but they are non-specific and non-adaptable, and do not evoke immunologic memory. Innate immunity is responsible for the acute antigen encounter, after which more specific responses of adaptive immunity take over, and the main role of innate immunity is to attenuate harm caused by pathogens and to restrict the spreading of the infections. The rapid reactivity of the effector cells of the innate immunity is made possible by the receptors that recognize pathogen-associated molecular patterns and are readily accessible on the surface of these cells.

Adaptive immunity, seen only in vertebrates having more advanced immunological system, allows the specific recognition of the non-self structures via antigen presentation, thus enabling identification of each pathogen by its specific antigenic "signature". Antigen recognition results in precisely targeted immune responses and development of immunologic memory, by which a later encounter with the same pathogen leads to a stronger and more swift and efficacious immune responses (Murphy et al. 2008).

CELL-MEDIATED AND HUMORAL IMMUNITY

Cell-mediated immunity refers to immune responses requiring cell-to-cell contacts, whereas humoral immune responses are mediated by the components of the liquid sections of the living organism, such as antibodies, cytokines or the complement system. Cell mediated immunity relies on antigen presenting cells (APCs) that introduce processed antigens attached to their surface receptors (HLA Class I and II molecules). The most important of APCs are the dendritic cells that are able, together with macrophages and B lymphocytes, to activate naïve CD4+ T cells. HLA Class II molecules are expressed by all nucleated cells and therefore activated CD8+T cells can be reactivated by all nucleated cells. Antibodies, mainly produced by B cells that have turned to plasma cells, are gamma globulin proteins (immunoglobulins) that are involved in the neutralization of pathogens and toxins, activation of the complement system (the classical activation pathway), opsonization (preparing pathogens for elimination), and phagocytosis (Murphy et al. 2008).

SELF-TOLERANCE AND AUTOIMMUNITY

Self-tolerance is maintained by active regulatory processes occurring both in locations producing new T cells (thymus) and B cells (bone marrow), and in the peripheral tissues. The central tolerance mechanisms aim at destroying or inactivating autoreactive lymphocytes, and the peripheral mechanisms at regulating the activity of autoreactive lymphocytes that have escaped the central negative selection. Although the majority of self-reacting T cells is deleted in thymus, continuously a small number of such lymphocytes enter the circulation and reach the peripheral tissues. In optimal conditions these autoreactive cells are suppressed by regulatory T cells, and thus remain inactive, but this self-tolerance can be broken, leading to initiation of an autoimmune process (Christen and von Herrath 2004a, Bluestone et al. 2008).

Although the mechanisms by which the autoimmune destruction is initiated and driven further have been studied intensively, the full picture of the course of events is still missing. It has been suggested that self-originating antigens share molecular characteristics (molecular mimicry) with antigens originating from foreign agents, i.e. viruses. The false recognition of the self-antigens might trigger the autoimmune process. A part of this false antigen recognition might be caused by genetic defects in the coding of the autoantigens and/or the antigen presenting molecules, which could result in an ineffective presentation of the autoantigens and suboptimal activation of regulatory cell responses. The spreading of the destructive process could occur via activation of the previously inactive nearby cells by signaling from the affected and dying cells ("by-stander effect"), which could join the destruction via apoptosis, and thus increase further the self-antigen load available (Fujinami et al. 2006).

One theory regarding the etiology of autoimmunity suggests that the disturbed balance between the Th1 responses (involved in the direct cell destruction) and Th2 responses (resulting in autoantibody production) could explain both allergies (Th2-based overactivation) and autoimmunity (Th1-based overactivation) (Christen and von Herrath 2004b, Sia 2005). According to this hypothesis, promoting the restoration of Th1/Th2

balance with immunomodulatory therapy, autoimmunity can be suppressed and normal self-tolerance regained. However, after several more or less successful experiments with immunomodulatory therapies targeted at modifying the Th1/Th2 balance, and by the more profound knowledge on the role of immunoregulatory cells, it seems that the Th1/Th2 model is not sophisticated enough to explain the complexity of immunotolerance. Briefly, today it seems more likely that maintaining normal self-tolerance requires adequate levels of normally-structured circulating autoantigens that can activate regulatory T cells to suppress the function of self-reacting T cells (Roep 2003).

PATHOGENESIS OF TYPE 1 DIABETES

CELLULAR AUTOIMMUNITY OF TYPE 1 DIABETES

Although there are several unanswered questions in theories regarding the molecular pathogenesis of T1D, the concept that cellular immune responses are responsible for the immune-mediated beta-cell destruction has been established. This view is supported by the observations that T cells are present in the islets of Langerhans in insulitis, immunosuppressive drugs selectively targeting T cells are able to delay the disease process, and beta-cell specific autoreactive T cells are present in the circulation of patients with newly diagnosed T1D (Roep 2003).

The site, the cause, and the primary autoantigen, if there is such an antigen, of the initial activation of the islet-specific autoreactive T cells have remained unidentified (Wong 2005). The activation of these T cells requires antigen presentation by MHC Class II molecules that are not normally expressed on beta cells, but can be induced *in vitro* with the combined stimulation of IFN-γ and TNF-α (Pujol-Borrel et al. 1987). These observations suggest that the initiation of the immune response targeting beta cells can take place in the islets. Yet, a more likely location for the activation is pancreatic lymph nodes, where APCs normally present antigens to naïve T cells.

The strong association between the genes coding for the MHC Class II molecules and T1D susceptibility supports the hypothesis that the beta-cell specific autoimmune response is antigen-driven. However, it is possible that autoimmunity against beta cells could originally represent autoantigen-independent hyperactivation of the immune system, triggered by cell damage caused by e.g. viral infection or toxic agents. This initial immune response could then disturb normal immune regulation and lead to breakage of self-tolerance. The primary autoantigen initiating the autoimmune process in T1D has remained unknown, although in the HLA DR4-DQ8-positive form of T1D it has been suggested to be either insulin or proinsulin (Eisenbarth 2003, Jasinski and Eisenbarth 2005, Wong and Wen 2005), while in the HLA DR3-DQ2-positive form of the disease, the 65 kD isoform of GAD could be the primary autoantigen, since the appearance of autoantibodies against this molecule has been associated with this haplotype (Knip 2002). The quest of the primary autoantigen still continues.

The next steps in the process leading to beta-cell damage are also partly undefined, but it seems that after priming, activated T cells migrate into the islets where they re-encounter their beta-cell specific autoantigen, become re-activated and cause inflammation in

the islets. The scarcity of suitable material for studies on the morphology of human pancreatic islets and the T1D-related changes in islet structure and function emphasize the significance of animal studies.

In non-obese diabetic (NOD) mice, inflammation in structures surrounding the islets (periinsulitis) (O'Reilly et al. 1991) precedes insulitis characterized by infiltrations of CD8+ and CD4+ T cells, macrophages, and B cells in the islets. The involvement of the CD4+ and CD8+ T cells in beta-cell destruction seems essential, since genetically athymic or perinatally thymectomized NOD mice do not develop autoimmune diabetes (Yoon and Jun 2001, Christen and von Herrath 2004a). According to studies with T cells that were transferred from diabetic NOD mice to neonatal or irradiated NOD mice, CD4+ T cells were able to accelerate independently the progression to diabetes in young recipients, but an effective transfer required the simultaneous transfer of CD8+ T cells reacting with MHC I molecules (Miller et al. 1988, Roep 2003, Wong and Wen 2005). The role of CD4+ cells is believed to be related to successful homing of CD8+ effector cells to the islets. The actual mechanisms of beta-cell death are not fully understood, but according to the first, recognition-based model, self-reactive cytotoxic T lymphocytes (CTLs) recognize autoantigens located on the surface of beta cells and induce cell death by secreting soluble death mediators, such as IFN-γ, TNF-α, IL-6, and free radicals. In the second, activation-based model, APCs located close to the islets introduce beta-cell specific antigens to T cells that become activated and cause apoptosis of the nearby beta cells through soluble mediators (Mathis et al. 2001).

The main problem with animal studies is that the results obtained are seldom applicable to humans as such, and often the simple phenomena observed in animals appear to be more complicated in man. Antigen-specific T-cell responses related to T1D have been studied mainly by using human peripheral blood mononuclear cells (PBMCs), and until recently, similar responses have been observed both in diabetic patients and healthy subjects. However, in a recent study with fresh PBMCs, both the classical proliferative T cell assay and an immunoblotting assay could distinguish patients from controls, indicating that a reasonable numbers of beta-cell specific autoreactive T cells do circulate in peripheral blood (Seyfert-Mangolis et al. 2006). Whether and how these cells reflect the autoimmune process in the islets remains still unknown.

Other open questions are related to the immunoregulation and the role of the regulatory T cells. It has been suggested that defects in this subpopulation of T cells might contribute to the initiation and/or maintenance of self-destructive autoimmune processes in T1D, and that, in theory, it could be possible to therapeutically boost immunoregulation by altering the functions of these cells (Tree et al. 2006, Roep 2003). It is, however, still too early for major clinical implications in this field.

HUMORAL BETA-CELL AUTOIMMUNITY

Immunoglobulins, like many other molecules, are able to permeate the placenta and reach the fetal circulation. Therefore, positivity for diabetes-associated autoantibodies during the first months of life can originate from the mother. In the case of maternally transferred autoantibodies both the maternal blood sample and the cord blood sample of the newborn

infant show similar autoantibody patterns, and without endogenous seroconversion (no signs of *de novo* synthesis), autoantibody levels decrease and disappear at the latest by the age of 18-24 months (Koczwara et al. 2004, Hämäläinen et al. 2000 and 2002).

In seroconversions occurring during the first years of life, IAA are often the first autoantibody reactivity to appear, followed by ICA, GADA and IA-2A, respectively, but any of the diabetes-related autoantibodies can be the first one to emerge (Kimpimäki et al. 2001a, Kupila et al. 2002, Kukko et al. 2005). The characteristics of autoantibody response (number, level, subclass and affinity of autoantibodies, as well as the number and types of epitopes) have been used in the prediction of T1D (Elliot and Pilcher 1994, Franke et al. 2005). In general, the broader the response (multiple positive DAAs with high levels and affinities, and multiple epitopes and subclasses), the higher the disease risk.

Affinity of the autoantibodies

Antigens may contain several binding sites and the strength of the binding between antigen and its receptor can vary remarkably. In general, strong binding of an antigen has been associated with advanced or mature immune responses. Affinity describes the binding force of a single antigen binding site and a single antigenic determinant, and is expressed as the association constant (k; litres/mole). Instead of representing the actual binding force of one actual interaction, it represents the average binding force of several similar, simultaneously occurring interactions. When the antigen is polyvariant, i.e. has several variable binding sites, the total strength of binding between a polyvariant antigen and its antibody is the sum of all the affinity bonds, and this total binding force is called avidity (Murphy et al. 2008). Affinity of autoantibodies has been regarded as a marker of maturation status of the immune response. Low affinity values have been reported to associate with transient and/or single autoantibody positivity and low disease risk (Wabl et al. 1999, Westerlund et al. 2005, Mayr et al. 2007, Schlosser et al. 2005a, Achenbach et al. 2007).

Epitope specificities, isotypes and subclasses of the autoantibodies

As the adaptive immune system communicates via T cell receptors, their epitope recognition is crucial for the development and loss of self-tolerance. Both intramolecular (from one epitope to another within the same autoantigen) and intermolecular (from one autoantigen to another) spreading of the autoimmune response is characteristic of prediabetic autoimmune response (Mackay and Rowley 2004, Di Lorenzo et al. 2007), and the spreading of humoral autoimmune response occurs mainly within a relatively short time frame (Hoppu et al. 2004a and b, Schlosser et al. 2005b, Kawasaki et al. 1998 and 2001). If the humoral autoimmune response has not spread within 12 months after the first signs of beta-cell autoimmunity, it rarely does so thereafter (Kupila et al. 2002).

According to observations in several prospective studies on the natural history of T1D, positivity for solitary autoantibody specificity represents mainly harmless non-progressive beta-cell autoimmunity (Colman et al. 2000, Mrena et al. 2003), whereas

the appearance of multiple autoantibodies reflects a progressive prediabetic autoimmune process that seldom reverts (Kulmala et al. 1998, Strebelow et al. 1999, Achenbach et al. 2004a). Transient positivity for diabetes-associated autoantibodies is a relatively rare phenomenon, both among young children with affected family members and in young children in the general population having HLA-conferred disease susceptibility (Spencer et al. 1984, Kimpimäki et al. 2002). Such positivity is, however, occasionally observed, especially among older siblings of affected children, in males, and in those who do not carry the high-risk HLA DR3/DR4 genotype (Yu et al. 2000a, Savola et al. 2001).

Various cytokines regulate the repertoire of immunoglobulin isotypes and subclasses, but the network of their action is complex and incompletely understood. In general, it has been proposed that as the active cytokine profile directs the class swiching of autoantibodies, likewise the isotypes of the antibodies present in peripheral blood may reflect polarization of immune responses towards either Th1- or Th2-biased responses (Mosman and Sad 1996).

AUTOANTIBODIES IN TYPE 1 DIABETES

Islet cell autoantibodies, ICA

Islet cell antibodies (ICA) were characterized in 1974 in diabetic patients with autoimmune polyendocrine deficiencies (Bottazzo et al. 1974, MacCuish et al. 1974). ICA are directed against cytoplasmic structures and are mainly of IgG (IgG1) class. They recognize all cell types in the pancreatic islets. Later studies on the epidemiology of T1D have shown that low-level (<20 JDFUs) ICA without other signs of beta-cell autoimmunity represent innocent, non-progressive beta-cell autoimmunity that is quite commonly detected among healthy relatives of patients with T1D, as well as in the general population (Bottazzo et al. 1991, Colman et al. 2002). High levels of ICA, meanwhile, are considered to reflect ongoing beta-cell destruction, since the frequency of the individuals with high ICA levels decreases sharply during the first years after the diagnosis of T1D, indicating that by the destruction of the islets also the target antigens have mainly disappeared. High levels of ICA are mainly detected along with other diabetes-predictive autoantibodies, resulting in multipositivity that is associated with an increased disease risk and short diabetes-free survival time (Bingley et al. 1996 and 1997, Mrena et al. 1999, Krischer et al. 2003).

The frequency of autoantibody positivity in patients with newly diagnosed T1D varies according to the age at diagnosis and the HLA risk genotypes of the diabetic individuals, and in Finland ICA positivity was observed to range from 30% (in individuals diagnosed earliest at the age of 20 years and carrying the HLA-DQB1*02/y genotype) to approximately 85% (in individuals diagnosed before the age of 15 and carrying the HLA-DQB1*0302/x genotype) (y and x represent non-protective alleles; Knip et al. 2002).

ICA do not represent a single autoantibody reactivity, but various antibodies that react with GAD_{65}, IA-2A, and other still unknown antigens (Månsson et al. 2001). Notably, ICA may not contain any reactivity related to IAA. These findings have been obtained

in experiments using preabsorption of ICA-positive sera with either insulin, GAD65 or the IA-2 molecule; the latter two block or decrease the subsequent ICA staining by sera containing these reactivities. Accordingly, ICA levels correlate relatively strongly with the IA-2A levels and more weakly with the GADA levels. The ICA titer can be considered to reflect the combined levels of IA-2A and GADA. Since ICA are detected with indirect immunofluorescence on sections of human pancreatic tissue from subjects with blood group O, the assay is difficult to standardize, is labor intensive, and requires access to high-quality human pancreatic tissue.

Autoantibodies against insulin, IAA

Antibodies against insulin detectable before treatment with exogenous insulin were discovered in 1983 (Palmer et al. 1983), and soon after that finding antibodies to proinsulin (PAA) were characterized (Castaño et al. 1993, Williams et al. 1999). IAA appear usually as the first of diabetes-associated autoantibodies, and although the IAA levels may fluctuate during the prediabetic phase, most often IAA positivity is of high affinity already at the beginning of the disease process, and thus rarely transient (Schlosser et al. 2005a, Achenbach et al. 2007).

In general, the prevalence of the IAA positivity has been reported to be 0.9-3.3% in the general population, while 40-60% of patients with newly diagnosed T1D are IAA positive at the time of diagnosis. IAA levels have been suggested to be predictive of T1D and to be highest among individuals with a rapid beta-cell destruction and progression to T1D (Yu et al. 2000b, Achenbach et al. 2004a). Genetic susceptibility to T1D, especially DR3/DR4 heterozygosity and DR4 homozygosity as well as the INS and PTPN22 gene polymorphisms associated with increased disease susceptibility, have a predisposing effect on the appearance of IAA positivity and the IAA levels (Walter et al. 2003, Hermann et al. 2005 and 2006). As described above, in Finland IAA positivity observed at diagnosis of T1D ranged from 20% (in individuals diagnosed earliest at the age of 20 years and carrying the HLA-DQB1*02/y genotype) to 60% (in individuals diagnosed before the age of 15 and carrying other HLA genotypes than those associated with high or moderate disease risk) (Knip et al. 2002).

Autoantibodies against glutamic acid decarboxylase, GADA

Glutamic acid decarboxylase (GAD) is an enzyme that catalyzes the formation of the γ-aminobutyric acid (GABA, an inhibitory neurotransmitter) from glutamate. GABA has been suggested to have a regulatory role in the secretion of insulin, glucagon, and somatostatin (Sorenson et al. 1991). The GAD protein has two molecular forms, GAD_{65} and GAD_{67} that differ from each other by molecular weight and repertoire of antigenic determinants. Antibodies against GAD (GADA) were first detected in 1982 as antibodies against the 64 kD antigen in patients with newly diagnosed T1D, and later also in patients with stiff-man syndrome (Baekkeskov et al. 1990, Daw et. al 1996). The 64 kD antigen was identified as GAD in 1990.

GADA are more common in postpubertal prediabetics and in patients with LADA, but depending on the population observed, 20-90% of newly diagnosed patients with T1D are

GADA-positive at the time of diagnosis (Seissler et al. 1993, Hagopian et al. 1995). Many initially GADA-positive diabetic patients remain positive for years after their diagnosis. This phenomenon has been suggested to occur due to the fact that GAD is expressed also outside the islets, thus providing a possible source of an activating autoantigen for GAD-targeted autoimmune responses (Ronkainen et al. 2004). In Finland GADA positivity observed at diagnosis of T1D ranged from 50% (in individuals diagnosed earliest at the age of 20 years and carrying the HLA-DQB1*02/y genotype) to approximately 70% (in individuals diagnosed before the age of 15 and carrying the HLA-DQB1*02/y genotype) (Knip et al. 2002).

Autoantibodies against islet antigen 2, IA-2A

In further studies on the 64 kD islet cell protein, it was shown that trypsin digestion of this molecule resulted in several protein fragments (50 kD, 40 kD, and 37 kD molecules) with different autoantibody binding profiles. The 40 kD fragment was recognized to be the intracellular portion of islet antigen 512, a transmembrane protein belonging to the family of protein thyrosine phosphatases (PTPs). This molecule is more commonly known nowadays as islet antigen 2, IA-2 (Rabin et al. 1994, Bonifacio et al. 1995a, Morgenthaler et al. 1997). Several potential islet-related antigens belonging to the family of PTPs have been identified, among them the precursor molecule of the 37 kD fragment, IA-2β (phogrin; Kawasaki et al. 1998). Antibodies against IA-2 (IA-2A) recognize the cytoplasmic domain of the IA-2 molecule, and no reactivity to extracellular regions of the molecule has been observed.

IA-2A appear usually as the last reactivity of the diabetes-related autoantibodies, and are thus considered as an indicator of advanced beta-cell autoimmunity, but occasionally the appearance of IA-2A has been the first sign of T1D-associated humoral autoimmunity. In the majority of these cases the appearance of IA-2A has been followed by wide-spread beta-cell specific autoimmune responses (Kimpimäki et al. 2002). In Finland IA-2A positivity observed at diagnosis of T1D ranged from 20% (in individuals diagnosed earliest at the age of 20 years and carrying the HLA-DQB1*02/y genotype) to approximately 95% (in individuals diagnosed before the age of 15 and carrying the HLA-DQB1*0302/x genotype) (Knip et al. 2002).

Autoantibodies against zinc transporter 8 (ZnT8A)

The ZnT8A, i.e. autoantibodies against the cation efflux transporter zinc transporter 8 (ZnT8) are the latest of the diabetes-associated autoantibodies that seem to gradually establish their role in the prediction of T1D. Zinc is a trace mineral that is supposed to participate in the formation of the hexamer insulin complexes (insulin storage molecule) found in insulin secretory granules. The beta-cell specific ZnT8 protein permits active efflux of zinc into the secretory granules (Chimienti et al. 2004).

Both the genetic variation of the gene encoding ZnT8 (SLC30A8) and the autoantibodies to ZnT8 have been suggested to have prognostic value in T1D (Wenzlau et al. 2007, Achenbach et al. 2009). The binding of the ZnT8A has been shown to correlate with the variants of the SLC30A8 gene. Subjects positive for ZnT8A (COOH terminal associated)

and homozygous for SLC30A8 SNP rs 13266634 are likely to progress to overt T1D more rapidly than subjects being heterozygous for this variant (Achenbach et al. 2009). The importance of ZnT8A is in the stratification of T1D risk, since about 60-70% of newly diagnosed patients test positive for ZnT8A at the time of diagnosis, and moreover, ZnT8A positivity has been observed both in prediabetic subjects and in patients with T1D who were previously identified as autoantibody negative or had tested positive only for single autoantibody reactivities (Gohlke et al. 2008, Achenbach et al. 2009). The combination of the analysis of ZnT8A and SLC30A8 genotyping may contribute to the prediction of T1D in the future.

EPIDEMIOLOGY AND ETIOLOGY OF TYPE 1 DIABETES

INCIDENCE OF TYPE 1 DIABETES

The incidence of T1D varies conspicuously between different countries. According to the WHO DIAMOND report, published in 2006 covering the time period 1990-1999, the difference between a high-incidence country like Finland (with an incidence of 40.9/100,000/year in children under the age of 14) and a low-incidence country such as China or Venezuela (with 0.1/100,000/year) was 400-fold (Soltez et al. 2007). The incidence of T1D has continuously increased almost everywhere in the world since World War II, and at the moment, the most rapid increase is observed in countries with rapid socio-economical development, such as Poland, the former Yugoslav Republic of Macedonia, Czech Republic, and Israel (Gale 2002, Patterson et al. 2009, Wild et al. 2004). Part of this increase is, however, associated with the fact that T1D is diagnosed nowadays at a younger age than some decades ago, and actually, the incidence of T1D in postpubertal subjects has gradually decreased according to some data sources (DIAMOND 2006, Patterson et al. 2009).

PREDIABETIC DISEASE PROCESS

The clinical presentation of T1D is usually preceded by a prediabetic phase associated with immune-mediated destruction of beta cells of the islets of Langerhans (Fig. 1). The first humoral signs of beta-cell autoimmunity appear often during the first year(s) of life, e.g. in the Finnish DIPP Study the youngest children to seroconvert were less than 3 months old (Kimpimäki et al. 2001a), but seroconversion to positivity for DAAs can occur at any age, as is the case with overt T1D. The prediabetic phase is highly variable in duration, ranging from months to decades, and especially among young IAA-positive children the disease process seems to be rapid and aggressive (Knip 2002).

The first detectable signs of beta-cell autoimmunity are the diabetes-associated autoantibodies, four of which, islet cell antibodies (ICA) and autoantibodies against insulin (IAA), the 65 kD isoform of the glutamic acid decarboxylase (GADA), and the tyrosine phosphatase-related IA-2 molecule (IA-2A) are nowadays used in the prediction of the disease risk. In many pediatric populations with genetic predisposition for T1D the proportion of the autoantibody positive individuals is higher than the prevalence of T1D (e.g. in Finland 4-6% and 1 %, respectively) indicating that even though signs of beta-

cell autoimmunity are seen, counteracting mechanisms do occur during the prediabetic phase and self-tolerance can be regained after the autoimmune process has once been initiated (Gardner et al. 1999, Kukko et al. 2005).

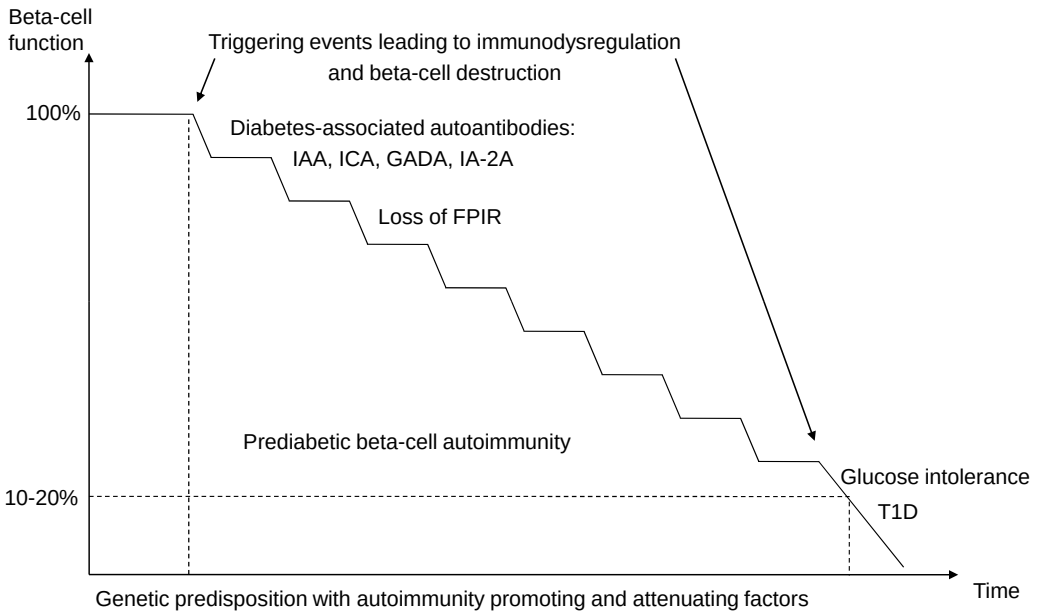

Figure 1. Progression to type 1 diabetes.
Modified from Eisenbarth 2003.

GENETIC BACKGROUND OF TYPE 1 DIABETES

A recent genome-wide association study combined with a meta-analysis of previous studies has shown that there are more than 40 genetic polymorphisms conferring susceptibility to T1D (Barrett et al. 2009). The majority of the susceptibility genes are encoding molecules that are involved in the immune responses. It has been shown that HLA region associated with disease risk (IDDM1) explains ~50% of the genetic disease susceptibility (Grant and Hakornason 2009) and all other regions associated with the disease susceptibility have a minor impact, a fact that is reflected by the estimated odds ratios for disease risk which are >6 for the HLA region and 1.1-2.4 for non-HLA genes. Although the impacts of the other single genetic determinants are minor, their combined effect may play a role in the initiation of the diabetic autoimmune process, especially among those with HLA genotypes associated with lesser disease susceptibility. Their role may also become more important in the future, as it seems that proportion of individuals with newly diagnosed T1D carrying neutral or protective HLA genotypes is gradually increasing (Fourlanos et al. 2008).

Table 1. HLA-DQB1 genotypes in 560 Finnish children with type 1 diabetes (T1D) and in 10541 healthy Finnish newborns. OR, odds ratio; PPV, positive predictive value.

HLA-DQB1 genotype	**Children with T1D, N (%)**	**Newborn infants, N (%)**	**OR**	**PPV***
Risk genotypes:				
02/0302	161 (28.8)	303 (2.9)	13.63	7.03
0302	191 (34.1)	1039 (9.9)	4.73	2.55
0302/0603	17 (3.0)	213 (2.0)	1.52	1.12
0301/0302	23 (4.1)	293 (2.8)	1.50	1.10
02	102 (18.2)	1384 (13.1)	1.47	1.04
Subtotal	*494 (88.2)*	*3232 (30.7)*		
Protective genotypes:				
02/0301	9 (1.6)	321 (3.0)	0.52	0.40
302/0602	6 (1.1)	324 (3.1)	0.34	0.26
02/0603	4 (0.7)	273 (2.6)	0.27	0.21
0301	10 (1.8)	1049 (10.0)	0.16	0.14
02/0602	2 (0.4)	406 (3.9)	0.09	0.07
0301/0603	1 (0.2)	198 (1.9)	0.09	0.07
0602	4 (0.7)	1421 (13.5)	0.05	0.04
0602 or 0603	3 (0.5)	1264 (12.0)	0.04	0.03
0301/0602	0 (0.0)	349 (3.3)	0.00	0.00
Subtotal	*39 (7.0)*	*5605 (53.2)*		
Others:	27 (4.8)	1740 (16.2)	0.26	0.22
Total	*560 (100)*	*10541 (100)*		

* Based on an estimated disease risk of 0.75% in the population studied.
Modified from Ilonen et al. 2002.

HLA genotypes

The observation that autoimmunity-based diseases aggregate in families holds true also for T1D, and the highest genetic disease risk is associated with the HLA genotype HLA-DQB1*02/*0302 (high-risk genotype), followed by HLA-DQB1*0302/x (x≠protective allele; moderate risk associated genotypes). The disease risk by the age of 15 years associated with the high risk genotype (frequency 3% in the Finnish background population) is ~7%, while the disease risk associated with the moderate risk genotypes (carried by 11% of this population) is 2-3% (Ilonen et al. 1996 and Hermann et al. 2004). The HLA region (IDDM1) associated with increased genetic susceptibility to T1D is located on the short arm of chromosome 6 (6p21, the MHC Class II region).

The dysregulation of the immune system associated with HLA is hypothesized to be related to changes in the antigen-binding peptide groove of the MCH Class II molecules,

which could have an effect on the binding affinity of the self-originating structures and could change the conformation of the antigen-MHC complex (Hermann et al. 2003). These changes in turn could make the self-antigen look foreign to T cells that are interacting with the HLA molecules, or generate strong immune responses that could lead to suppression of the regulatory T cell responses (Caillat-Zucman 2009). Different HLA genotypes have been associated with several autoimmune diseases, e.g. HLA-B27 with spondylarthropathies, and HLA-DR3 and/or HLA-DR4 with T1D, celiac disease, Addison's disease, myasthenia gravis and autoimmune thyroid diseases (De Block et al. 2001).

Insulin gene (INS -23 HphI) polymorphism

The gene coding for insulin is positioned on the short arm of chromosome 11 (11p15), and its role as a genetic determinant of T1D was reported for the first time as early as in 1984 (Permutt et al. 1984). The INS gene is associated with a variable number of tandem repeats (VNTRs) that can be classified into three groups: Class I containing 30-60 repeats (high-risk variant), Class II containing 60-120 repeats (slightly protective variant), and Class III containing 120-170 repeats (protective variant). The number of repeats is associated with the amount of insulin produced in thymus, and thus presented to maturating T cells. Higher concentrations of thymic insulin may induce effective negative selection of insulin-reactive T cells, and the increased risk for T1D that is associated with the homozygous genotype of Class I is attributed to the low concentrations of thymic insulin available (Walter et al. 2003). The effect of Class II and III alleles is, however, dominant, and only one of these protective alleles is needed to ensure the protective effect (Haller et al. 2004, Barrat et al. 2004).

Protein tyrosine phosphatase-like molecule 22 (PTPN22) gene polymorphism

The gene for the protein tyrosine phosphatase-like molecule 22 (PTPN22), a negative regulator of T cell activation, is located on the short arm of chromosome 1 (1p13). A single-nucleotide polymorphism (SNP; C1858T; Arg620Trp variant) in this gene has been associated with a series of autoimmune diseases, including T1D, systemic lupus erythematosus (SLE), rheumatoid arthritis, autoimmune thyreoid diseases, and generalized vitiligo (Vang et al. 2007, Gregersen and Olssen 2009). The high-risk and moderate-risk variants (TT and CT, respectively) have been reported to participate in the regulation of insulin-specific autoimmunity and progression from prediabetes to clinical disease, and they associate with an increased risk for developing positivity for IAA and additional autoantibodies. The effect of PTPN22 on disease susceptibility seems to be more significant in males and in subjects with low-risk HLA genotypes (Hermann et al. 2006).

Cytotoxic T-lymphocyte antigen 4 (CTLA-4) gene polymorphism

The gene for the cytotoxic T-lymphocyte antigen 4 (CTLA-4) is located on the short arm of chromosome 2 (2q33), and polymorphism (SNP +49 A/G) of this gene causes changes in the levels of intracellular CTLA-4 and IL-2, which in turn leads to altered T-cell proliferation. These changes may contribute to the initiation/progression of beta-

cell autoimmunity by decreasing the levels of CTLA-4 in CD4+ and CD4+CD25+ cells, or decreasing the number of Treg cells. Polymorphism of CTLA-4 has been associated with T1D, autoimmune thyroid diseases, celiac disease, and SLE. CTLA4 is expressed on the surface of regulatory T cells and activated effector T cells, and its role as the key down regulator of the T cell function has been emphasized by the fact that mice lacking this gene (CTLA-4 knockout mice) will die young from a severe lymphoproliferative disorder resulting from aggressive autoimmunity (Schmidt et al. 2009). However, the association with an increased risk of T1D has been modest and not confirmed in all studies (Ueda et al. 2003, Hermann et al. 2005).

Autoimmune regulator gene (AIRE)

The autoimmune regulator gene (AIRE) is located on the short arm of chromosome 21 (21p22), and loss of function mutations of this gene result in the development of a recessively inherited disorder called autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED or autoimmune polyglandular syndrome type 1, APS-1), which is characterized by loss of self-tolerance in multiple organs. The protein coded by AIRE regulates the expression levels of peripheral self-antigens in the thymus, such as insulin, and the prevalence of T1D among APECED patients is relatively high (~18%) (Gylling et al. 2000, Holmdahl 2007).

GENETIC VS. ENVIRONMENTAL FACTORS IN ETIOLOGY OF TYPE 1 DIABETES

Several facts indicate that environmental factors propably play critical roles in the etiology of T1D and genetic factors can not explain all phenomena seen in the epidemiology of T1D. Some of the most prominent arguments in favor of a crucial contribution of environmental determinants are the following:

1. In most populations with available data the incidence of T1D has increased remarkably after World War II, e.g. in Finland from 12/100 000/year in 1953 to 64/100 000/year in 2006 among children under the age of 15 years (Harjutsalo et al. 2008). Changes in the genetic pool occur slowly over many generations.

2. There are significant differences in the incidence of T1D between populations with highly similar genetic backgrounds but different living conditions, e.g. a six-fold gradient between Finland and Russian Karelia (Kondrashova et al. 2007).

3. The concordance of T1D is around 50% among monozygotic twins (Redondo et al. 2001, Metcalfe et al. 2001).

4. Genes predisposing to T1D are relatively common in the general population, e.g. in Finland the combined proportion of risk conferring HLA genotypes is approximately 20% in the general population, and still less than 5% of individuals with such genotypes will eventually develop T1D (Ilonen et al. 2002). Even individuals with a protective genotype may occasionally develop T1D (Table 1).

5. The incidence of T1D increases in the offspring when people move from a low-incidence country to a high-incidence country (Hjern and Söderström 2008).

As the major burden of T1D lies upon countries with high standard of living, located in the northern part of the globe, factors that are related to the climate or the amount of sunshine, the socio-economic structure of society, the level of education and the standard of hygiene have been implicated to explain the phenomenon. So far none of these factors have been shown to have a primary role in the development of T1D, but interestingly, even in a low-incidence country like China, climate has been observed to have an effect on the disease risk; the incidence of T1D is higher during the winter months and in the colder areas of China (Yang et al. 2005). The seasonal variation in the appearance of beta-cell autoimmunity and overt T1D, with a peak incidence occurring during the winter months in the northern hemisphere, has been observed in many countries, e.g. in Hungary (Gyürüs et al. 2002), the northern part of the United Kingdom (Mooney et al. 2004), and Belgium (Weets et al. 2004).

Also the month of birth has been linked to the disease risk in various parts of the world (Lewy et al. 2008, Kahn et al. 2009). These observations suggest that environmental factors operating during the prenatal and postnatal development of the child may be important in the etiology of T1D. The mechanisms by which various environmental factors mediate their actions resulting in the initiation and progression of the prediabetic disease process have largely remained unknown. The reason for this confusing picture is most likely that each risk factor requires optimal timing and dosing, repeated exposures and co-acting determinants to have an effect on the risk of developing T1D.

Microbial etiology

In humans, the well-know connection between the congenital rubella infection and an increased prevalence of diabetes in the offspring represents the most solid evidence for the involvement of microbial agents in the initiation of the beta-cell destruction (Yoon 1985, Gale 2008). Other indirect evidence includes the seasonal variation in the appearance of the first diabetes-associated autoantibodies and in the manifestation of clinical T1D resembling the variation in the frequency of certain common virus infections (Kordonouri et al. 2002, Moltchanova et al. 2009). Furthermore, enterovirus-positive staining has been detected in histological sections of islets of patients that have died soon after the diagnosis of T1D (Oikarinen et al. 2008).

Theories regarding the mechanisms by which viral infections could lead to the initiation of beta-cell autoimmunity and destruction include: 1) molecular mimicry, whereby parts of foreign antigens and self-originating molecules might have similar antigenic structures leading to cross-reactivity with the self-antigen instead of the pathogenic antigen; 2) the hygiene hypothesis, according to which early infections facilitate the development of a balanced immune system, and a reduced microbial load during the early years of life may increase the risk of autoimmunity; 3) the “bystander effect”, in which beta cells undergoing destruction caused by a viral infection could send activation signals to initially unaffected nearby cells, which would then join the destructive process and worsen the cell damage, exposing further autoantigens to be presented to autoreactive T cells (von Herrath 2009).

Bacterial, fungal, and parasitic exposures provided by environment may also have an effect on risk for T1D, most probably mainly by driving immune responses towards Th2-dominated responses and normal self-tolerance. However, the role of fungal infections is less well known and there are indications that fungal toxins might even contribute to the risk of diabetes by causing stress to endoplasmic reticulum (Hettiarachchi et al. 2008). As the incidence of T1D has increased rapidly since the World War II, we might learn important lessons about the role of hygiene and living conditions in the development of T1D by studying the longitudinal changes in these factors.

Another approach to study factors associated with the hygiene hypothesis is to study societies with similar genetic disease susceptibility background but differing living conditions and social standard as well as incidence of T1D. One natural geographic area for this kind of studies lies in the north-eastern Europe covering Finland, Estonia and North-Western Russian Karelia. Estonia represents a country in which similar social and economic changes that Finland went through after WWII have occurred during the last decades, and in Russian Karelia there are areas in which the standard of living resembles that of Finland in the 1950's. The genetic pool is relatively similar in these three countries, but the incidence of T1D in Finland is nearly three-fold when compared to Estonia and six-fold when compared to Russian Karelia (Kondrashova et al. 2007, Teeäär et al. 2009). This study design has been utilized in an ongoing study, the DIABIMMUNE project, in which the hygiene hypothesis and the mechanisms related to the standard of hygiene that could potentially explain changes in immunoregulation are tested in the context of T1D and other immune-mediated diseases.

Nutritional factors

General health and the nutritional status of the pregnant woman is directly reflected in the well-being of the fetus, and factors that regulate the transplacental transfer of nutrients, antigens and antibodies to the fetus could theoretically have an effect on the risk of T1D in the offspring. Effects of nutritional prenatal conditions on subsequent risk of T1D have been reported in several studies. Only one association has been established: inverse correlation between maternal cod liver oil supplementation (containing vitamin D, vitamin A, and omega-3 fatty acids) during pregnancy and risk of T1D in the offspring (Stene et al. 2004). Studies on the effects of postnatal nutrition have, in contrast, resulted in several important findings that might possibly be applied in future trials aimed at preventing T1D. These findings are related mainly to the key nutritional elements of the first year of life, such as breastfeeding, supplemental feeding, and vitamin D supplementation (Åkerblom et al. 2005, Wahlberg et al. 2006, Holmberg et al. 2007, Rosenbauer et al. 2008).

The role of early feeding in the initiation of autoimmunity against islet cells has remained controversial. According to results from the TRIGR trial studying infants with at least one affected family member, the risk of beta-cell specific autoimmunity was lower among infants who had received a highly hydrolyzed formula as to compared to infants fed with a regular formula (Åkerblom et al. 2005). From the Finnish DIPP study it was reported that short duration of exclusive breastfeeding and early introduction of supplementary

formula feeding were related to an increased risk of advanced beta-cell autoimmunity (Kimpimäki et al. 2001b).

The mechanisms by which breastfeeding might mediate its protective effects against T1D comprise protection against infections via protective (IgA class) antibodies secreted into breast milk, delayed exposure to foreign dietary antigens, and enhancement of the maturation of the gut-associated lymphoid tissue caused by cytokines and growth factors present in breast milk (Piirainen et al. 2009). Bovine insulin present e.g. in ordinary cow milk-based formula has been shown to induce production of anti-bovine insulin antibodies that are cross-reactive with human insulin, thus being a potential inductor of immune responses against endogenous insulin in the infant (Tiittanen et al. 2006, Vaarala 2006).

In the German BabyDiab study, however, no association was observed between cow's milk exposure and beta-cell autoimmunity, whereas early (age <3 months) introduction of cereals was associated with an increased risk of beta-cell autoimmunity, when compared to later introduction of cereals (Ziegler et al. 2003). Also an American study indicated that early exposure to cereals (before the age of 4 months) was a risk factor for subsequent beta-cell autoimmunity, but in that study also late exposure, at the age of 7 months or later, increased the risk of beta-cell autoimmunity (Norris et al. 2003).

Various vitamins, minerals, and fatty acids such as vitamin D and E, nicotinamide (vitamin B3), zinc, and n-3 fatty acids (eicosapentaenoic acid and docosahexaenoic acid) have been suggested to protect against T1D (Elliot and Chase 1991, Knekt et al. 1999, Mandrup-Poulsen et al. 1993, Manna et al. 1992, Haglund et al. 1996, Uusitalo et al. 2008). The active form of vitamin D (1,25-dihydroxyvitamin D) has been shown to increase the mRNA levels of interleukin-4 (IL-4) and transforming growth factor and to decrease the concentrations of INF-γ and TNF-α mRNAs, thus inducing a deviation in the function and activity of T helper cells (Cantorna et al. 2004, Mathieu et al. 2004). Vitamin D deficiency has been linked to an increased risk of T1D in epidemiological studies, but since this condition is rare in developed countries, the protective effect of vitamin D supplementation is difficult to prove in the general population. The optimal dosing of vitamin D is also poorly defined, and the doses for supplementation in infancy have varied from a daily dose of 200 IU in the USA to 400 IU in Finland to 100,000 IU used in a French study (Lamberg-Allardt and Viljakainen 2008).

The promising results obtained from animal studies regarding the protective effect of nicotinamide was an impetus for the European Nicotinamide Diabetes Intervention Trial (ENDIT) in which 549 first-degree relatives of patients with T1D who were ICA-positive ($\geq$20 JDFU) were randomized to receive either oral nicotinamide 1.2 g/m^2/day (ad 3 g/day) or placebo for 5 years. Nicotinamide supplementation did not prevent T1D, although it reduced the level of INF-γ in high-risk individuals (Schatz and Bingley 2001, Gale et al. 2004, Hyppönen 2004).

In general, reports of associations between nutritional factors and the risk of T1D have not been convincing. This is not surprising when taking into account that these studies have focused on nutritional factors in common use that may act as predisposing

determinants only in genetically susceptible individuals (Atkinson and Gale 2003). So far there are some implications that breastfeeding, nicotinamide, zinc, and vitamins C, D, and E provide some degree of protection against T1D, while early exposure to cow's milk, obesity, increased linear growth, and N-nitroso compounds may increase the risk (Virtanen and Knip 2003, Moltchanova et al. 2004). As any single nutritional factor may have only a weak effect, it is challenging to obtain firm evidence for the significance of a specific factor in the etiology of T1D.

Early growth and body composition

Studies focusing on growth-related risk factors have resulted in contradictory findings. The risk for T1D associated with increased energy intake (and thus with high-energy nutrients) has been hypothesized to be due to metabolic disturbances, insulin resistance and compensatory increase in the expression of insulin induced by obesity, but already high or low birth weight for gestational age have been associated with an increased disease risk for T1D. Rapid weight gain in early infancy has been associated with an increased risk for T1D in several case-control studies (Hyppönen et al. 2000, Harder et al. 2009). Increased relative height has also been linked to an increased risk of T1D in some studies, but results from other studies have been contradictory (Wilkin 2001, Harder et al. 2009).

INSULIN: SYNTHESIS, SECRETION AND RESISTANCE

The synthesis of insulin that occurs in the pancreatic beta cells begins with the synthesis of the insulin precursor molecules, preproinsulin and proinsulin. After initial synthesis proinsulin is cleaved by proteolytic enzymes into three protein chains, A, B, and C. The C-peptide is then removed and the A chain is bound to the B chain via disulfide bonds to form the active insulin molecule. Since equal amounts of active insulin and C-peptide are produced, the measurement of C-peptide can be used to assess the patient's endogenous insulin secretion after the initiation of treatment with exogenous insulin. After posttranslational modifications insulin is packed into insulin secretory granules located near the plasma membrane of the beta cell and is then ready to be secreted (Becker et al. 2001).

The secretion of insulin is triggered by a rise in the blood glucose level that also increases the amount of glucose molecules entering the beta cells, which in turn causes the release of secretory granule-packed insulin. This initial secretion phase, first phase of insulin release (FPIR), begins 1-2 minutes after the initiation of the glucose stimulus and continues for 10-15 minutes. Intravenous glucose stimulates insulin secretion less efficiently than oral glucose administration, because oral nutrients trigger also the mediatory signaling of the gastrointestinal tract, resulting in the release of peptides that enhance insulin secretion, such as glucose-dependent insulinotropic peptide, cholecystokinin, and glucagon-like peptide-1 (Mari et al. 2008).

The second phase of insulin release, which requires synthesis and secretion of newly produced insulin molecules, begins when the glucose stimulus continues. FPIR promotes peripheral utilization of glucose, suppresses hepatic gluconeogenesis, and

limits postprandial glucose elevation. The binding of insulin to its peripheral receptors initiates a cascade that leads to translocation of the glucose transporter molecules to the plasma membranes of the target cells and increased glucose uptake to these cells. Without appropriate insulin stimulus, peripheral cells use other sources than glucose for their energy production, which results in the formation of acidic metabolic end-products (ketones), and if the insulin deficiency continues, the buffer capacity of the body is exceeded at some point, and the state of ketoacidosis is reached (Faideau et al. 2005).

In T1D the loss of beta-cell mass begins months to years before the appearance of clinical signs of diabetes, and at the time of diagnosis, 80-90% of the beta cells have been destroyed. Frequently, the remaining beta cells are dysfunctional and respond poorly to glucose stimulation (Keskinen et al. 2002, Barker et al. 2007). The fading of FPIR represent an early sign of a disturbed beta-cell function that appears soon after seroconversion, at a time when no other abnormalities in the glucose metabolism can be observed (Bingley et al. 1992, Colman et al. 1998, Mrena et al. 2003, Harrison 2001). The mechanisms maintaining the balance between beta-cell replication/neogenesis and apoptosis/destruction is unknown in humans, but prolonged hyperglycemia and elevated levels of free fatty acids have been suggested to be toxic to beta cells (glucotoxicity and lipotoxicity) (Maedler et al. 2002, Martin-Gallán et al. 2007).

The programming of insulin resistance, a condition in which a normal amount of insulin results in an incomplete insulin response in peripheral tissues, has remained poorly defined. It seems that factors appearing already in the prenatal environment may predispose to insulin resistance, since individuals born small for gestational age are more insulin resistant later in life than those with higher birth weight and normal early weight gain (Veening et al. 2002 and 2003).

The sensitivity to insulin follows the pattern of normal distribution, and is significantly reduced in early puberty (Conwell et al. 2004). Although body composition plays an important role in insulin sensitivity and an increased insulin resistance can be observed in pregnancies associated with excessive weight gain and in obese patients with metabolic syndrome, it also partly explains the transient hyperglycemias observed in various infections and can be seen even in cachectic patients suffering from anorexia nervosa (Scheen et al. 1988, Conwell et al. 2004).

In general, in individuals with normal glucose metabolism, beta cells can compensate the increase in insulin resistance by enhancing insulin secretion, and this enhancement can be observed both in FPIR and in basal insulin secretion. Insulin resistance alter the metabolism of various target organs, resulting to reduced glucose uptake and storing of local glycogen in the muscle cells, impaired synthesis of glycogen and unnecessary active gluconeogenesis in the liver cells, and enhanced hydrolysis of triglycerides leading to an increased release of free fatty-acid from the adipose tissues. The increase in the levels of circulating glucose and free fatty-acids might explain the reduction of beta-cell mass occurring also in advanced T2D in which no immune-mediated islet cell destruction is observed (Becker et al. 2001).

The accelerator hypothesis

Insulin resistance has been hypothesized to link together the two major types of diabetes, T1D and T2D. This hypothesis originates from the findings that the incidence rates of obesity and T1D are rising simultaneously, and that sometimes it is clinically difficult to distinguish between T1D and T2D at the time of diagnosis. According to the accelerator hypothesis (Wilkin 2001) there are three factors that accelerate beta-cell destruction: obese bodily constitution, insulin resistance, and autoimmune response targeting islet cells. The accelerator hypothesis implies that none of the accelerators can lead to diabetes in the absence of weight gain, which causes an increase in insulin resistance and weakens the control of glucose metabolism. The elevated blood glucose level could in turn accelerate apoptosis of the beta cells via glucotoxicity, and the increased beta-cell immunogenicity would further accelerate apoptosis and lead to an intense immune response in a subset of genetically predisposed individuals. "Only tempo distinguishes type 1 from type 2" (Wilkin 2001). The accelerator hypothesis has been tested in young patients with newly diagnosed T1D and the findings have been controversial. Some recent studies have reported no connections between body composition, indicated by relative weight, and progression to T1D (Dabalea et al. 2006, O'Connell et al. 2007).

Assessment of insulin resistance

Several methods for assessing beta-cell function and insulin resistance have been reported. The "gold standard" has been the hyperinsulinemic euglycemic clamp technique in which insulin is infused intravenously in increasing concentrations (rate 10-120 mU/m^2/minute) and the amount of infused glucose (20% solution) needed to maintain euglycemia (blood glucose 5.0-5.5 mmol/l) is measured. The higher the amount of glucose needed to prevent hypoglycemia, the higher the insulin sensitivity of the subject. This technique requires, however, complicated methodology and frequent sampling, and is also time-consuming, and therefore more simplified methods have been developed.

Two methods based on the fasting values of glucose and insulin, the Homeostasis Model Assessment for Insulin Resistance (HOMA-IR = $[Glucose_{fasting}]$ x $[Insulin_{fasting}]$ / 22.5) and the Quantitative Insulin-Sensitivity Check Index (QUICKI = 1/ {log$[Insulin_{fasting}]$ + $[Glucose_{fasting}]$}), have been demonstrated to provide insulin resistance/sensitivity values that correlate well with the values obtained from the clamp method (Matthews et. al 1985, Katz et al. 2000) and also have a good intercorrelation (Radziuk 2000). By combining the fasting data with a short intravenous glucose tolerance test in which glucose (0.5 g/kg, 20% solution) is intravenously infused within 3 minutes ± 15 seconds and venous blood samples for glucose and insulin assessments are taken 1, 3, 5, and 10 minutes after the infusion, both the assessment of insulin resistance and FPIR can be simultaneously obtained.

Regarding pediatric population, the major hindrance for utilizing the data gathered from IVGTTs has been the absence of reference values obtained from non-obese, healthy children and adolescents. Lately, the reported thresholds for insulin resistance (HOMA-IR) in adolescents have ranged from 3.2 to 4.4 (Lee et. al 2006).

PREDICTION AND PREVENTION OF TYPE 1 DIABETES

PREDICTION OF TYPE 1 DIABETES

By 2004, when the current work was initiated, the predictive characteristics of the susceptibility genes, diabetes-related autoantibodies, and FPIR had been established in the FDR population (Tables 1, 2, and 3; Bonifacio et al. 1995a, Greenbaum et al. 1999, Mrena et al. 1999, Bingley et al. 2001), while the knowledge of the role of these markers in the general population had been scarce but accumulating.

Table 2. Data on the four major diabetes-associated autoantibodies available by 2003.

Antigen	Detection	Relevance to prediction	Examples of references
Islet cells	Indirect immunofluorescence on sections of human pancreas	High ICA level associated with increased risk of diabetes Disease risk associated with intracellular staining pattern	10, 22, 23, 30, 33, 34, 35, 38, 76, 80, 125, 137, 153, 169, 186, 208, 246
Insulin	Radiobinding assay using specific mono-iodinated insulin	IAA first to appear in prediabetes in children Distinct epitopes might identify diabetes-related from non-progressive IAA	30, 44, 80, 84, 144, 194, 211, 259, 274, 275, 294
GAD65	Radiobinding assay using radiolabelled human recombinant GAD65	Prevalence of GADA increase with age and is higher in females Middle and carboxy-terminal regions are the major antigenic regions Epitope recognition conformational Predicts the future need for exogenous insulin in type 2 diabetic subjects	2, 10, 11, 19, 27, 41, 54, 64, 86, 128, 132, 207, 227, 228, 247, 250, 252
IA-2 (40-kD)	Radiobinding assay using radiolabelled recombinant IA-2	IA-2A positivity in multipositive relatives predict rapid progression to type 1 diabetes Intracellular domain the main immunodominant region Epitope recognition conformational Shared antigens between IA-2 and IA-2β (37-kD)	27, 35, 36, 48, 56, 151, 155, 158, 162, 190, 195, 198, 202, 211, 218, 231, 268, 283, 292

Modified from Franke et al. 2005.

Table 3. Prevalence of the four major diabetes-associated autoantibodies in the general population and in first-degree relatives of patients with type 1 diabetes.

Autoantibodies	Prevalence (%), general population	Prevalence (%), first-degree relatives	Examples of references
ICA	0.59-5.3	1-12	25, 33, 34, 50, 57, 63, 78, 91, 121, 125, 137, 143, 146, 149, 196, 210, 217, 221, 243, 257, 278, 286
IAA	1.07-3.9	1.4-6.9	22, 25, 27, 50, 79, 80, 91, 121, 124, 143, 146, 196, 210, 226, 257, 262, 286
GADA	0.4-2.97	5-13	2, 11, 18, 19, 27, 35, 79, 86, 91, 121, 146, 149, 196, 243, 262, 286
IA-2A	0.1-2.4	1.5-5.3	19, 25, 74, 79, 121, 146, 149, 196, 198, 202, 210, 217, 243, 262, 286

Modified from Franke et al. 2005.

The focus of the prediction of T1D has been on the unaffected family members of patients with T1D, since their identification is relatively easy due to the regular medical follow-up visits needed for the index case, and because the disease risk is approximately 3- to 5-fold when compared to individuals of the general population. However, most (~90%) patients with newly diagnosed T1D have no affected family members, and targeting only FDRs of affected patients will result in the identification of the minority of individuals with an increased risk for T1D.

The Bayes' theorem claims that predictive markers have lower positive predictive value in the general population than in a selected group of individuals among whom the prevalence of the outcome measures is higher, i.e. FDRs of patients with T1D (Díaz et al. 2003). Accordingly, single predictive factors provide rarely enough information on the disease risk for subjects in the general population, but the combination of several risk factors may lead to similar disease risk estimates that are observed for family members of affected patients.

Today the basis of prediction of T1D in the general population lies on the identification of subjects carrying genetic T1D susceptibility and regular autoantibody monitoring of the genetically T1D-prone individuals. According to observations in the FDR population, the next step in the stratification of the disease risk is to assess the metabolic status of those individuals that develop signs of beta-cell autoimmunity during the follow-up. By combining immunologic and metabolic markers, the assessment of the disease risk in susceptible individuals in the general population can reach the level required for the recruitment of subjects for trials aimed at disease prevention. The ultimate purpose

of prediction of T1D is not only to clarify the natural history of T1D, but to develop effective measures aimed at preventing beta-cell destruction caused by the immune-mediated response targeting the beta cell.

PREVENTION STRATEGIES

The accumulated knowledge of the immunoregulatory mechanisms involved in the development of T1D has paved the way for the development of immunomodulatory means of preventing T1D and although no break-through has been seen yet, several potentially successful intervention trials are ongoing. Prevention schemes can be classified into four different stages according to the diabetic disease process (Fig. 2): primary prevention targeting genetically susceptible individuals without any signs of beta-cell autoimmunity; secondary prevention during prediabetic beta-cell autoimmunity; tertiary prevention at the time of the diagnosis of T1D; late prevention of disease-related complications and attempted restoration of the lost functions in an established disease.

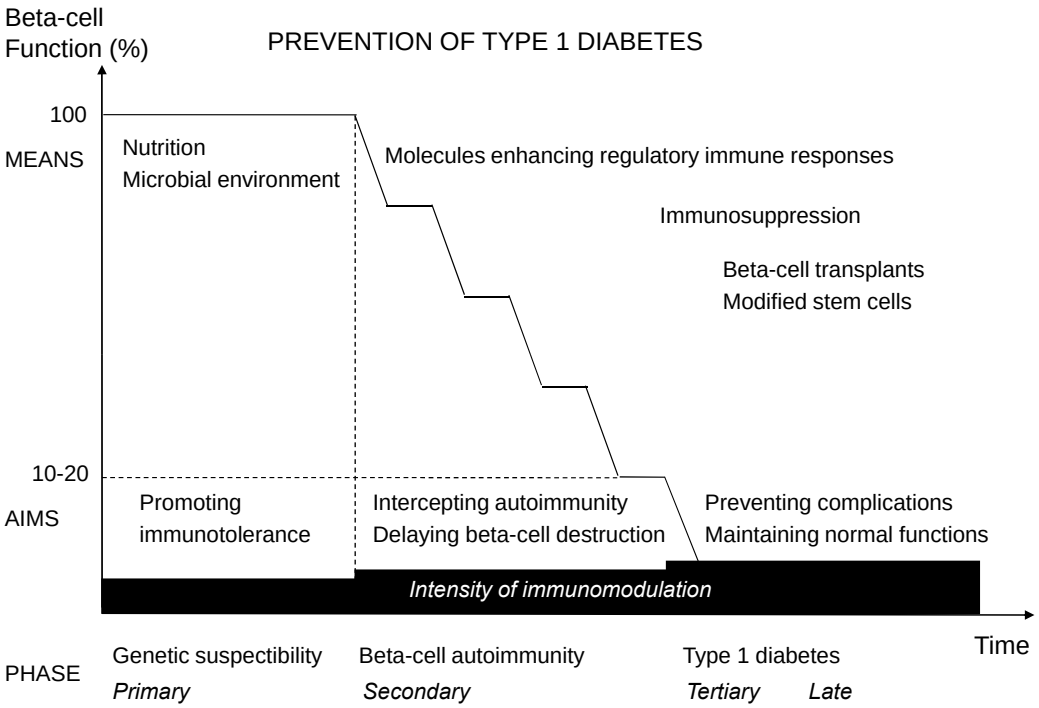

Figure 2. Time line for prevention of type 1 diabetes.

An example of primary prevention is the Trial to Reduce IDDM in the Genetically at Risk (TRIGR), in which genetically susceptible infants with affected family members are weaned to either a normal cow's milk-based formula or to a highly hydrolyzed formula containing fragmented milk proteins instead of intact, more immunogenic ones (Åkerblom et al. 2005). The latest ongoing secondary prevention trials include a study with oral insulin given to prediabetic individuals with high IAA levels (DPT-1), and another study

in which humanized FcR non-binding anti-CD3 monoclonal antibody will be given to prediabetic subjects (Schatz and Bingley 2001, Bisikirska and Herold 2004, Skyler et al. 2005). Tertiary prevention trials aimed at preserving the beta-cell mass and its remaining function, and if possible, promoting its recovery, are performed on patients with newly diagnosed T1D, and as these patients already have established T1D, more effective, but potentially more harmful immunomodulatory treatments might be used. The latest ongoing tertiary prevention trials include studies with parenterally administrated GAD (Diamyd®), CTLA-4 Ig (Abatacept®), anti-CD20 (Rituximab®), a rabbit polyclonal anti-thymocyte globulin (Thymoglobulin, Thymo®), and IL-2 (Proleukin®) combined with rapamycin (Rapamune®). The majority of these intervention studies are organized within the TrialNet network (http://www.diabetestrialnet.org; Skyler 2008).

ETHICAL CONSIDERATIONS

An important fact supporting the establishment of a population-based screening program is that the clinical status of newly diagnosed T1D patients has been shown to be better among those who have partaken in prospective follow-up before diagnosis (Barker et al. 2004, Hekkala et al. 2007). However, each step of the screening program aimed at the identification of individuals with an increased disease risk may potentially cause psychological discomfort and ethically challenging situations. Even the decision to take part in genetic screening may cause anxiety, especially since the testing occurs often during puerperium, when the mothers of the newborn infants are highly sensitive to any concerns regarding their offspring. The role of the personnel informing the families and individuals about the screening procedures is critical and good communication skills are essential.

The psychological effects of the screening programs have been studied in parallel with ongoing programs. According to these studies, the participating families are in general coping well with the mental pressure caused by the study procedures (Roth 2001, Ludvigsson et al. 2001, Simonen et al. 2006). According to a Finnish study on families participating in the DIPP study, more than 90% of the parents were content with the knowledge regarding the disease risk, even though 55% of the mothers and 37% of fathers of the high-risk newborn infants had experienced modest anxiety when they had received the results of the genetic screening. In families experiencing increased discomfort, anxiety was usually connected with other stressful life events, and the coping mechanisms in the family were emotion-focused or based on avoiding behavior (Simonen et al. 2006). However, while planning a screening program, it is important to pre-arrange a strategy to identify individuals and families with anxiety and to provide them with more intensive counseling, if needed. Adaptation to the fact of being at increased genetic disease risk, and if applicable, to the prediabetic disease process and to overt T1D may become easier, if problematic issues are discussed during the follow-up period.

AIMS OF THE CURRENT STUDY

To evaluate the value of DAA and metabolic determinants as prognostic factors in the prediction of T1D in children with and without family history of T1D we have:

1) Compared the predictive values of GADA and IA-2A and their combination between FDR of patients with newly diagnosed T1D and a comparable cohort of general population over a 15-year follow-up period.

2) Assessed the predictive characteristics of diabetes-associated autoantibodies in a general population-derived cohort of children with HLA-conferred susceptibility to T1D.

3) Assessed whether children who progress rapidly to T1D are characterized by a higher prediabetic IAA affinity than IAA-positive subjects remaining unaffected or progressing more slowly to T1D, and whether IAA affinity increases when the time of diagnosis approaches.

4) Assessed the role of the first-phase insulin release and insulin resistance as predictors of T1D in children with HLA-conferred disease susceptibility and signs of advanced beta-cell autoimmunity recruited from the general population.

RESEARCH DESIGN AND METHODS

STUDY SUBJECTS

PUBLICATION I

In this observational study, signs of beta-cell autoimmunity and T1D were assessed in two cohorts of healthy Finnish children and adolescents. The first cohort was derived from the Childhood Diabetes in Finland (DiMe) study, in which 755 non-diabetic siblings of children with newly diagnosed T1D were recruited at the time of diagnosis of the index child (Table 4, Tuomilehto et al. 1992). The second cohort comprised 3-18 year-old individuals (n=3475) from the general population that were randomly selected from the Finnish National Registry to take part in the Cardiovascular Risk Factors in Young Finns (LASERI) study (Table 4, Åkerblom et al. 1999). The comparisons between the two population cohorts were based on autoantibody measurements in single blood samples obtained as soon as possible after the identification of the index case in the sibling cohort, and at the first visit to the study center in the general population cohort.

Table 4. Study subjects in Publication I. Time-related variables are medians (range).

	DiME (n=755)	**LASERI (n=3475)**
Population cohorts	Siblings of children with newly diagnosed T1D	Random selection; 3-18 year-old individuals from the general population
Geographical coverage	All Finnish hospitals taking care of children with newly diagnosed T1D	Five university hospital referral areas in Finland
Recruitment	During 1986-1989	In 1980
Follow-up for the current report	December 31, 2002	June 30, 1995
Progression to T1D, n (%)	51 (6.8%)	15 (0.4%)
Age at diagnosis of T1D, years	14.1 (1.5-28.4)	16.8 (5.5-32.8)
Follow-up for non-progressors, years	14.8 (13.7-16.3)	14.9 (14.8-15.1)
Timing of the blood samples	The first sample obtained after the diagnosis of the index child	The first sample obtained
Age at sampling, years	10.0 (1.3-19.9)	10.8 (2.7-18.9)

PUBLICATION II

The Finnish Diabetes Prediction and Prevention (DIPP) study

In the Finnish Diabetes Prediction and Prevention (DIPP) study, children from the general population with genetic predisposition for T1D are observed from birth for

signs of beta-cell autoimmunity and clinical T1D. The DIPP study was launched in 1994 in Turku, in 1995 in Oulu and in 1997 in Tampere (Kimpimäki et al. 2002). The majority (>90%) of the 11 000 babies annually born in these centers participate in the cord blood screening to assess HLA-conferred susceptibility to T1D, and infants carrying the high risk genotype (HLA DQB1*02/0302) or the moderate risk genotypes (HLA DQB1*0302/x; x≠*02, *0301, *0602, or *0603) are invited to a prospective follow-up study. Publication II contains data on a subcohort of DIPP children (n=7410) who had participated in the follow-up at least until the age of 1 year by August 31, 2004, or had developed T1D by that time (Table 5; Publication II, Online Supplement Fig. 1). Data on diabetes-associated autoantibodies and progression to T1D was collected until December 31, 2008.

Table 5. Study subjects in Publications II-IV. Time-related variables are medians (range).

	Publication II DIPP cohort (n=7410)	Publication III DIPP subcohort (n=128)	Publication IV DIPP subcohort (n=218)
Population cohorts	Children with HLA-conferred susceptibility to T1D derived from the general population	IAA-positive DIPP children: 64 progressors and 64 matched unaffected children	Persistently multipositive DIPP children; Undergone at least one IVGTT by December 31, 2005
Geographical coverage	Turku, Oulu, and Tampere[a]	* Same as Publication II	*
Recruitment	Ongoing. Based on cord blood samples[b]	*	*
Follow-up for the current work	Until December 31, 2008	*	*
Timing of the blood samples	Every 3-12 months since birth	*	*
Data assessed in the current thesis work	All autoantibody samples available by December 31, 2008	First and last prediabetic/ early diabetic IAA-positive samples from progressors and comparable samples from non-progressors	Autoantibody samples available by December 31, 2008; weight and height (from birth to IVGTT); metabolic data from the first IVGTT
Progression to T1D, n (%)	180 (2.4%)	64 (50%)	117 (53.7%)
Age at diagnosis of T1D, years	5.0 (0.9−12.5)	3.9 (0.9−8.8)	5.3 (2.1–12.5)
Follow-up for non-progressors, years	9.3 (5.4−14.2)	7.6 (3.5−11.7)	10.2 (4.9–14.2)

[a]University referral areas in Finland; [b]Recruitment started in Turku (1994), followed by Oulu (1995) and Tampere (1997)

In the DIPP study, the information regarding the family history of T1D is generated with structured questionnaires completed by the parents soon after the birth of each index child. In the Oulu and Tampere study centers, clinical follow-up visits are scheduled to take place at the age of 3, 6, 12, 18, and 24 months, and after that annually, while in Turku the schedule for the basic visits is once in every 3 months until the age of 2 years, and thereafter with an interval of 6 months. Follow-up visits are arranged in all centers every 3 months for children who develop signs of beta-cell autoimmunity (seroconversion to ICA positivity; See chapter Autoantibodies). Infants with transplacentally acquired maternal antibodies are regarded as seronegative as long as no *de novo* synthesis of diabetes-related autoantibodies is observed. In this report autoantibody positivity was considered to be persistent (prefix "p") if at least two sequential samples (taken at least 3 months apart) and the last sample available were positive for the same autoantibodies. The last prediabetic and/or the first diabetic samples (obtained within 7 days after the diagnosis) were taken into account when defining the persistence of the autoantibody status.

Intervention Trial with Nasally Administrated Insulin

Subjects with persistent positivity for at least two of the four autoantibodies measured (ICA, IAA, GADA, and IA-2A) were eligible for an intervention trial with intranasally administrated insulin. Before starting the treatment, eligible children underwent oral (OGTT) and intravenous glucose tolerance tests (IVGTT) to exclude subclinical diabetes and to assess their glucose metabolism. The aim in the intervention was to delay the clinical manifestation of T1D, but unfortunately, as the treatment group codes were opened in November 2007, the results showed that this type of intervention did not have any effect on the progression rate to T1D (Näntö-Salonen et al. 2008). For participation in the intervention trial and its effects, see: Publication II, Supplementary Table1; Publication III, Tables 2-3; Table 15.

PUBLICATION III

Pilot studies 1-2 for Publication III

The method of measuring IAA affinity was optimized in two pilot studies on 57 samples from the Finnish Pediatric Diabetes Register before starting the analyses for Publication III (Table 6, Mäkinen et al. 2008). In the first part of the pilot study, samples obtained from diabetic patients (n=17) and from unaffected family members (n=14) of patients with T1D were studied, while in the second part samples obtained from unaffected parents (n=24) and siblings (n=17) of patients with newly diagnosed T1D were analyzed. In addition, some samples from siblings who were unaffected at initial sampling, but later progressed to T1D (n=7) were studied.

Table 6. Study subjects in the pilot studies for Publication III. Time-related variables are medians (range).

	Pilot study 1	**Pilot study 2**
Source	The Finnish Pediatric Diabetes Register (n=57)	
Selected subjects	Patients with parenteral insulin treatment for T1D (n=17)	Unaffected parents (n=24) and siblings (n=17) of patients with T1D
	Unaffected family members of patients with T1D (n=14)	Initially healthy siblings that later progressed to T1D (n=7)
Geographical coverage	All Finnish hospitals treating new patients with T1D	* Same as in Pilot Study 1
Recruitment	At the time of the diagnosis of the index case in the family; sample collection started in 2002	*
Blood samples in focus	The first samples taken at the diagnosis of the index child	*
Progression to T1D, n (%)	7 (14.6%)	*
Age at sampling, years	27.3 (1.4−58.6)	

By December 31, 2006, 118 children (1.6%) from the original DIPP subcohort (described above) had developed clinical T1D (Table 5). Repeated positivity for IAA was observed in 82 (69.5%) of these progressors in preclinical phase. Based on the availability of samples for the assessment of IAA affinity, 64 IAA-positive progressors and 64 non-diabetic IAA-positive control children from the original DIPP cohort (non-progressors) were included in the study. The non-progressors were matched with the progressors for HLA genotype, gender, age at the appearance of IAA positivity (±12 months), place of birth, and the availability of subsequent IAA-positive samples. Regarding gender, HLA genotype, and age at the appearance of IAA positivity, the proportion of completely matched pairs was 92%. For detailed data on the study subjects, see Publication III, Tables 1-3.

PUBLICATION IV

As described above, DIPP children older than 1 year of age with persistent positivity for multiple autoantibodies and a non-diabetic OGTT were eligible for an intervention trial with intranasally administrated insulin, and before starting the intervention treatment these children underwent an IVGTT (Publication IV, Table 2 and Fig. 1). The study cohort in this work comprised 218 DIPP children with results from at least one IVGTT by December 31, 2005, and 179 (82.1%) of these children participated in the intervention trial.

METHODS

GENETIC SCREENING

The HLA-DQB1-associated risk genotypes (HLA DQB1*02/0302 and HLA DQB1*0302/x; x≠*02, *0301, *0602, or *0603) were screened in cord blood samples

by time-resolved triple-label hybridization with allele-specific probes (Sjöroos et al. 1995). Insulin gene (INS; -23 HphI variant) polymorphism was assessed with a PCR-based method and lanthanide-labeled oligonucleotide hybridization, and the PTPN22 C1858T polymorphism with a homogenous genotyping method and a minisequencing assay (Haller et al. 2004, Hermann et al. 2006).

AUTOANTIBODY ASSAYS

Blood samples obtained from the two cohorts, DiMe and LASERI, were analyzed for GADA and IA-2A between the years 2002 and 2004 to ensure comparable antibody analyzing methods. According to the DIPP Study protocol, the measurement of ICA is used as the first step of autoantibody screening, and if a child seroconverts to ICA positivity or develops diabetes, also the other three autoantibodies, IAA, GADA, and IA-2A, are analyzed in all samples available from that individual. A subset of DIPP-children (n=1006) was directly screened for all the above mentioned autoantibodies.

Serum samples for autoantibody determinations were stored at −70ºC prior to analysis. ICA were measured by indirect immunofluorescence on sections of human pancreas (cut-off level for positivity 2.5 Juvenile Diabetes Foundation Units, JDFU; Bottazzo et al. 1974), and IAA, GADA, and IA-2A with specific radiobinding assays as described previously (Williams et al. 1997, Savola et al. 1998 a and b). Cut-off values for IAA (3.48 Relative Units, RU), GADA (5.36 RU; 14.13 WHO Units/ml), and IA-2A (0.43 RU; 1.91 WHO Units/ml) positivity were based on the 99th percentile levels observed in Finnish non-diabetic pediatric population (n=370). All ICA-positive samples and samples with IAA, GADA and/or IA-2A levels between the 97th and 99.5th percentiles were reanalyzed to confirm the antibody status.

The laboratories that performed the autoantibody measurements have participated in the international Diabetes Autoantibody Standardization Program (DASP) workshops. According to the workshop results in 2003, the disease sensitivity and specificity of the GADA assay were 82% and 98%, while the corresponding characteristics for the IA-2A assay were 64% and 100%, respectively. In 2005 the disease sensitivity of the IAA, GADA, and IA-2A assays were 58%, 82%, and 72%, respectively, while corresponding specificities were 98%, 96%, and 100%. Disease sensitivity and specificity of the ICA assay were 100% and 98%, respectively (Greenbaum et al. 1992a, Bingley et al. 2003, Törn et al. 2008).

ASSAY FOR MEASUREMENT OF IAA AFFINITY

Insulin autoantibody affinity was assessed with a competitive homologous radiobinding assay with human recombinant insulin (Amersham, GE Healthcare, Buckinghamshire, UK; mono-^{125}I labeled at TyrA14 with activity 2000 Ci/mmol and concentration 0.148 nmol/l) after competition with eight increasing concentrations (ranging from 4.0×10^{-13} to 5.9×10^{-5} mol/l) of unlabeled human recombinant insulin (Roche Diagnostics, Mannheim, Germany). The assay protocol was a modification of that described earlier (Achenbach et al. 2004b). The total volume of the samples (measured in duplicates) was 55 µl/well. The amount of bound labeled insulin was measured with a liquid scintillation detector

(1450 MicroBeta Trilux; Perkin Elmer Life Sciences, Turku, Finland) after precipitation of immune complexes, and the results were given as counts per minute (cpm). GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, CA, USA) was used to calculate the values for the half maximal inhibitory concentration (IC50) and dissociation constant (Kd), and the reciprocal of the Kd value (l/mol) was used to represent the IAA affinity. The coefficient of variation of the assay was 12.3%. Further details regarding the assay; see Publication III (Materials and Methods section).

ORAL AND INTRAVENOUS GLUCOSE TOLERANCE TESTS

Oral glucose tolerance tests were performed by giving perorally (p.o.) 1.75 g glucose/kilogram body weight (Glucodyne® 227.3 mg/ml solution, maximal dose 75 g) after taking the baseline (0 min.) venous sample for glucose measurement. Another sample for testing the plasma glucose level was taken 2 h after the administration of glucose, and the results were interpreted according to the WHO criteria (Table 7).

Table 7. Diagnostic criteria for diabetes mellitus and other types of hyperglycemia.

	Glucose concentration, mmol/l (mg/dl)		
	Whole blood		Plasma
	Venous	Capillary	Venous
Diabetes mellitus			
Fasting or	≥ 6.1 (≥ 110)	≥ 6.1 (≥ 110)	≥ 7.0 (≥ 126)
2h after glucose challenge	≥ 10.0 (≥ 180)	≥ 11.1 (≥ 200)	≥ 11.1 (≥ 200)
Impaired glucose tolerance			
Fasting, if measured, and	< 6.1 (<110)	< 6.1 (< 110)	< 7.0 (< 126)
2h after glucose challenge	≥ 6.7 (≥ 120)	≥ 7.8 (≥ 140)	≥ 7.8 (≥ 140)
Impaired fasting glycemia			
Fasting, and if measured	≥ 5.6 < 6.1 (≥ 100 < 110)	≥ 5.6 < 6.1 (≥ 100 < 110)	≥ 6.1 < 7.0 (≥ 110 < 126)
2h after glucose challenge	< 6.7 (< 120)	< 7.8 (< 140)	< 7.8 (< 140)

Modified from World Health Organization 1999.

The standardized ICARUS protocol (Bingley et al. 1992) was used for performing the IVGTTs: After overnight fasting, baseline samples for the glucose and insulin measurements were taken and 0.5 g glucose/kg body weight was infused intravenously (20% solution) in 3 minutes ± 15 seconds. The maximal dose of glucose was 35 g. Venous blood samples for glucose and insulin assessments were taken 1, 3, 5, and 10 minutes after the infusion. Glucose concentrations were measured in plasma samples with an enzymatic method, and serum intact insulin concentrations with an enzyme-linked two-site immunoassay (Beach and Turner 1958, Yalow and Berson 1960). Cross-reactivity with proinsulin was not tested. Insulin concentrations in serum samples obtained in Oulu and Tampere were analyzed in the Research Laboratory, Department of Pediatrics, University of Oulu, while the samples obtained in Turku were assayed in the laboratory of Turku University Hospital (Dako Cytomation, Ely, United Kingdom). The concentrations analyzed in Turku were converted to values comparable to those

analyzed in Oulu according to a regression equation (R^2=0.94) based on a comparison of 100 samples between Oulu and Turku.

The sum of the insulin concentrations in post infusion samples at 1 and 3 minutes was defined as the first-phase insulin response (FPIR), and the homeostasis model assessment of insulin resistance index (HOMA-IR) was calculated by multiplying the fasting glucose value (mmol/l) by the fasting insulin value (mU/l), and by dividing the result by 22.5.

GROWTH-RELATED DATA

The weight (kg) and height (cm) of the children participating in the DIPP study were measured at the clinical follow-up visits. The original values obtained were compared to the Finnish standardized growth charts (Sorva et al. 1984) and converted into relative height (a standard deviation score value, SDS) and relative weight (weight-for-height percentage, %).

DATA ANALYSIS

The SPSS program versions 11.0 to 16.0 (SPSS, Chicago, IL, USA) were used for the statistical analyses. The confidence intervals were given at 95% ($CI_{95\%}$) and statistical significance was set at P<0.05 (two-tailed), except in the case of multiple simultaneous analyses, where the Bonferroni correction was used, when applicable. Various parametric and non-parametric methods were used, the details of which are respectively described in the original publications.

CASE ASCERTAINMENT

In publication I, cases were ascertained from the Central Drug Registry of the National Social Insurance Institute (Karvonen et al. 1999). For DIPP-related studies, cases were ascertained from the patient records of the three University Hospitals participating in the DIPP study and from the Finnish National Pediatric Diabetes Register (Mäkinen et al. 2008).

ETHICAL ISSUES

The study protocols of the DiMe, LASERI, and DIPP studies have been approved by the local ethics committees, and written informed consents were obtained from the guardians of the participants before the commencement of the study procedures. In the DIPP study, results from HLA genotyping and autoantibody analyses were provided to the participating families together with medical consultation services, if needed. DIPP children who had developed persistent positivity for at least two of the diabetes-associated autoantibodies and had reached the age of 1 year were offered the possibility to participate in a double-blinded, randomized, placebo-controlled intervention trial with nasally administrated insulin. Regarding these children, written informed consents were obtained separately prior genetic analysis, and before initiating the follow-up period or intervention study.

RESULTS

Children who progress to T1D seroconvert at a young age

Comparable data on the initial seroconversion were unavailable for the DiME and DIPP cohorts, but according to data from the DIPP cohort, seroconversion occurring at a young age was a strong risk factor for T1D in children with an increased genetic disease risk. The current DIPP cohort comprised 7410 children that were observed for signs of beta-cell autoimmunity and progression to T1D for a median follow-up time of 9.2 years (range 0.9-14.2 years; Publication II, Supplementary Table 1). The median age at diagnosis of the 180 progressors (2.4%; 93 males) was 5.0 years (range 0.9-12.5 years), and the median follow-up time for the unaffected subjects 9.3 years (range 5.4-14.2 years). The age at seroconversion of unaffected ICA-positive subjects was 4.2 years (0.2-13.7 years), whereas the age of progressors was 1.5 years (0.3-9.6 years, $P<0.001$).

Children with the high-risk HLA genotype and/or positive family history of T1D seroconverted more frequently and were younger at seroconversion than DIPP children carrying moderate-risk genotypes and having no affected FDRs (Tables 8-9). High-risk genotype, positive family history for T1D and male gender were also associated with multipositivity appearing already at the time of initial seroconversion (Table 10). Correlations between seroconversion and HLA genotype and seroconversion and family history with T1D remained significant even after controlling for the other baseline factor (HLA genotype or family history of T1D, respectively; adjusted r_S= –0.07 and 0.05,

Table 8. Comparison between DIPP children carrying the high-risk HLA genotype and the genotypes associated with moderate disease risk.

	High risk	**Moderate risk**
	N (%)	
Total	1575 (21.3)	5835 (78.7)
Gender, males	829 (52.6)	3068 (52.6)
T1D affected family members at the birth of the child	53 (3.4)	124 (2.1)*
ICA-based seroconversion	325 (20.6)	848 (14.5)†
Seroconversion sample IAA-positive [a]	83 (25.5)	155 (18.3)‡
Seroconversion sample multipositive [a]	64 (19.7)	97 (11.4)†
Positivity for ≥2 DAAs [a]	141 (43.4)	250 (29.5)†
Persistent autoantibody positivity [a]	194 (59.7)	478 (56.4)†
Persistently positive for ≥2 DAAs [a]	109 (33.5)	174 (20.5)†
Participation in the intervention study	82 (25.2)	137 (16.2)†
Progression to T1D	80 (5.1)	100 (1.7)†
	Years, median (range)	
Age at diagnosis	5.1 (1.0–12.0)	4.9 (0.9–12.5)
Age at seroconversion	3.5 (0.3–13.3)	4.0 (0.2–13.7)§
Age at maximal DAA status	4.2 (0.5–13.3)	5.0 (0.5–13.7)‖
Delay from seroconversion to maximal DAA status	0 (0–9.0)	0 (0–11.0)*
Delay from seroconversion to diagnosis of T1D	2.8 (0.1–9.0)	2.8 (0.0–10.9)

[a]Among ICA-positive subjects; *P=0.004, †P<0.001, ‡P=0.006, §P=0.03, ‖ P=0.04.

Table 9. Comparison between DIPP children with and without first degree relatives with type 1 diabetes at birth of the child.

	FDRs	**No family history of T1D**
	N (%)	
Total	177 (2.4)	7233 (97.6)
Gender, males	83 (46.9)	3814 (52.7)
HLA-DQB1*02/*0302, high risk genotype	53 (29.9)	1522 (21.0)*
ICA-based seroconversion	49 (27.7)	1124 (15.5)†
Seroconversion sample IAA-positive [a]	23 (46.9)	215 (19.1)†
Seroconversion sample multipositive [a]	16 (32.7)	145 (12.9)†
Positivity for ≥2 DAAs [a]	38 (21.5)	353 (4.9)†
Persistent autoantibody positivity [a]	40 (22.6)	632 (8.7)†
Persistently positive for ≥2 DAAs [a]	31 (17.5)	252 (3.5)†
Participation in the intervention study	26 (14.7)	193 (2.7)†
Progression to T1D	24 (13.6)	156 (2.2)†
	Years, median (range)	
Age at diagnosis	4.8 (0.9–11.4)	5.1 (1.0–12.5)
Age at seroconversion	3.0 (0.3–10.5)	4.0 (0.2–13.7)‡
Age at maximal DAA status	4.0 (0.5–12.5)	4.9 (0.5–13.7)
Delay from seroconversion to maximal DAA status	0.5 (0–8.7)	0 (0–11.0)†
Delay from seroconversion to diagnosis of T1D	2.8 (0.3–8.6)	2.8 (0.0–10.9)

[a]Among ICA-positive subjects; *P=0.004, †P<0.001, ‡P=0.02.

Table 10. The effects of gender on seroconversion and type 1 diabetes-related disease process in the DIPP study cohort (N=7410).

	Males	Females
	N (%)	
Total	3897 (52.6)	3513 (47.4)
High risk genotype (HLA-DQB1*02/*0302)	829 (52.6)	746 (47.4)
T1D affected family members at the birth of the child	83 (2.1)	94 (2.7)
ICA-based seroconversion	621 (15.9)	552 (15.7)
Seroconversion sample IAA-positive [a]	148 (3.8)	90 (2.6)*
Seroconversion sample multipositive [a]	98 (15.8)	63 (11.4)†
Positivity for ≥2 DAAs [a]	235 (6.0)	156 (4.4)‡
Persistent autoantibody positivity [a]	380 (9.8)	292 (8.3)†
Persistently positive for ≥2 DAAs [a]	169 (4.3)	114 (3.2)§
Participation in the intervention study	130 (3.3)	89 (2.5)‖
Progression to T1D	93 (2.4)	87 (2.5)
	Years, median (range)	
Age at diagnosis	4.9 (0.9–12.5)	5.3 (1.0–12.0)
Age at seroconversion	4.0 (0.5–13.7)	4.0 (0.2–13.2)
Age at maximal DAA status	4.9 (0.5–13.7)	4.8 (0.5–13.5)
Delay from seroconversion to maximal DAA status	0 (0–10.0)	0 (0–11.0)§
Delay from seroconversion to diagnosis of T1D	2.8 (0.1–10.9)	2.9 (0.0–9.0)

[a]Among ICA-positive subjects; *P=0.001, †P=0.03, ‡P=0.002,§P=0.01, ‖ P=0.04.

respectively; $P<0.001$ for both), indicating that these two factors have an independent effect on the risk of seroconversion. Gender did not correlate with ICA-based seroconversion as such, but male gender was related to indicators of more advanced beta-cell autoimmunity, especially persistent positivity for multiple diabetes-associated autoantibodies (unpublished data).

The child's age at seroconversion predicted development of T1D. According to the analyses of diabetes survival, DIPP children who seroconverted before the age of 2 years had the highest cumulative disease risk (36.9%, $CI_{95\%}$ 28.5-45.3%; Publication II, Fig. 1A), and the odds ratio (OR) for T1D was 5.0 ($CI_{95\%}$ 3.5-7.1) when younger seroconverted subjects (age <2 years) were compared to those who had seroconverted after the age of 2 years. Subjects seroconverting before the age of 2 years were more often positive for multiple autoantibodies already at the time of the first positive sample than subjects who seroconverted later (18.3% vs. 12.1%, $P=0.006$), but the correlation between age at seroconversion and T1D remained significant after controlling for multipositivity (adjusted $r_S= –0.12$, $P<0.001$; unpublished data, Table 11).

DIPP children with the high-risk HLA genotype were younger at seroconversion than those with moderate-risk HLA genotypes. Similarly those with a family member affected by T1D were younger at seroconversion than those with a negative family history (Tables 8-9). However, young age at seroconversion correlated with the risk for T1D even after adjusting for the association between HLA genotype and FDR status (adjusted $r_S= –0.28$, $P<0.001$; unpublished data).

Table 11. Positivity for insulin autoantibodies (IAA) or for multiple autoantibodies at first ICA-positive sampling in relation to age at seroconversion among 1173 ICA-positive DIPP children. ICA, islet cell antibodies.

	N (%)	Age at ICA-based seroconversion (years)*			
		0-1.99	2-3.99	4-5.99	≥6
Seroconversion sample IAA-positive	238 (20.3)	132 (41.4)	64 (23.4)	27 (11.9)	15 (4.2)
Seroconversion sample multipositive	161 (13.7)	58 (18.2)	52 (19.0)	30 (13.2)	21 (5.9)

*$P\leq0.001$ in all comparisons between two consecutive groups

Age at seroconversion does not correlate with the pace of the prediabetic disease process

The initial hypothesis was that children who seroconvert at a young age will also progress to overt T1D more rapidly than their peers, but in the current DIPP study cohort and over the present observation period this was not the case (Fig. 3). The median delay from seroconversion to diagnosis was 2.8 years (0.02-10.9 years) in ICA-positive children who developed T1D, and the delay did not correlate with the seroconversion age ($r_S=0.005$, $P=0.95$; unpublished data). However, children who had seroconverted after the age of 6 years had a slightly faster disease progression than children with seroconversion during their third or fourth year of life. The difference in the progression

rate was most prominent during the first 2.5 years after the seroconversion, but on other hand the number of progressors was low in the eldest cohort, which most likely skews the results of this analysis.

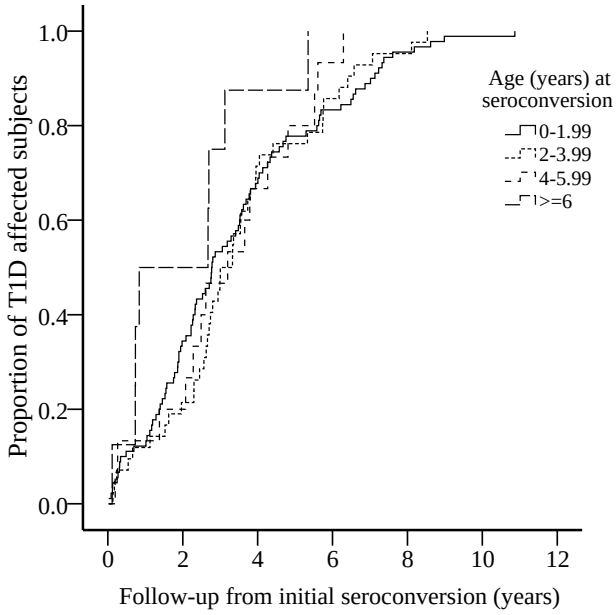

Figure 3. Effect of age at seroconversion on the delay from seroconversion to diagnosis of type 1 diabetes in DIPP children positive for at least ICA. *P*=0.04 between the second youngest and the oldest group of children.

The maximal effects of baseline factors (gender, HLA genotype, and family history of T1D) are seen in the initiation of the prediabetic disease process

The effects of the two main baseline factors, i.e. HLA genotype and family history of T1D, were apparent in the whole DIPP study cohort, especially for factors related to the beginning of the prediabetic disease process. In this study cohort of young children the gender-related differences in the autoantibody values were minor, and although markedly high GADA levels associated with the female gender, the mean GADA level was higher in males, both at initial seroconversion and during the follow-up (Table 12). The correlations between T1D and the levels of ICA, IAA, GADA, and IA-2A remained all significant after adjusting for HLA genotype, gender, and family history of T1D (unpublished data: r_S=0.29 for ICA, r_S=0.46 for IAA, r_S=0.22 for GADA, and r_S=0.33 for IA-2A; *P*<0.001 for all).

In DIPP children with advanced beta-cell autoimmunity, HLA genotype and FDR status played a minor role in terms of disease development, and statistically significant differences were observed only for GADA that appeared to be higher in children with the high-risk HLA genotype (Table 12). Higher GADA levels associated also with the female gender in these children.

Table 12. Autoantibody levels during the follow-up assessed by baseline factors in 7410 DIPP children and the subgroup of DIPP children with advanced beta-cell autoimmunity (n=283).

	HLA genotype		Family history with T1D		Gender	
	High risk	**Moderate risk**	**Positive**	**Negative**	**Males**	**Females**
Whole cohort:						
Initial seroconversion						
ICA	5 (3–640)	5 (3–668) *	7 (3–110)	5 (3–668) *	5 (3–320)	5 (3–668)
IAA	1.1 (0–93.0)	0.4 (0–167.8) *	5.3 (0–167.8)	0.5 (0–93.0) *	0.5 (0–93.0)	0.4 (0–167.8)
GADA	0.5 (0–1154.5)	0.2 (0–598.2) *	1.8 (0.1–342.1)	0.2 (0–1154.5) *	0.3 (0.0–598.2)	0.2 (0–1154.5) ||
IA-2A	0.1 (0.0–221.7)	0.1 (0.0–247.3) †	0.1 (0.0–38.2)	0.1 (0.0–247.3) §	0.1 (0.0–247.3)	0.1 (0.0–129.5)
At maximal DAA status						
ICA	5 (2–640)	5 (2–1742) *	12 (2–640)	5 (2–1742) *	5 (2–1742)	5 (2–668)
IAA	1.4 (0–180.8)	0.4 (0–213.1) *	6.1 (0–213.1)	0.5 (0–180.8) *	0.6 (0–213.1)	0.5 (0–180.8)
GADA	0.8 (0–1154.5)	0.3 (0–1009.4) *	6.4 (0.1–168.7)	0.3 (0–1154.5) *	0.4 (0–653.9)	0.24 (0–1154.5) ¶
IA-2A	0.1 (0.0–221.7)	0.1 (0.0–247.3) ‡	0.7 (0.0–128.1)	0.1 (0.0–247.3) *	0.1 (0.0–247.3)	0.1 (0.0–129.5) #
Children with advanced autoimmunity:						
Initial seroconversion						
ICA	4 (0–168)	5 (0–668)	5 (0–110)	4 (0–668)	4 (0–259)	5 (0–668)
IAA	6.1 (0–58.8)	7.0 (0–70.0)	6.2 (0–43.5)	6.6 (0–69.9)	6.5 (0–66.2)	6.5 (0–70.0)
GADA	6.1 (0.0–189.5)	1.9 (0.0–153.8) ∂	7.8 (0.1–153.8)	2.7 (0.0–189.5)	2.2 (0.0–189.5)	5.9 (0.1–172.3)
IA-2A	0.1 (0.1–93.2)	0.1 (0.1–121.0)	0.1 (0.1–13.0)	0.1 (0.1–121.0)	0.1 (0.1–96.4)	0.1 (0.1–121.0) †
Peak level before IVGTT						
ICA	44 (4–640)	44 (4–1742)	28.5 (5–168)	44 (4–1742)	44 (4–1742)	44 (4–1742)
IAA	9.8 (0–198.1)	12.6 (0–319.6)	10.1 (0–146.1)	12.3 (0–319.6)	12.4 (0–319.6)	9.6 (0–198.1)
GADA	58.3 (0.3–374.7)	20.1 (0.1–410.6) ∂	49.0 (0.6–410.6)	34.7 (0.1–374.7)	25.0 (0.1–374.7)	54.0 (0.1–410.6) #
IA-2A	2.6 (0.1–221.7)	21.2 (0.1–247.3)	0.8 (0.1–127.1)	17.9 (0.1–247.3)	21.2 (0.1–247.3)	8.9 (0.1–231.2)
At IVGTT						
ICA	28 (0–640)	29 (0–1742)	29 (2–168)	28 (0–1742)	23 (2–1742)	34 (0–1280)
IAA	7.4 (0–194.4)	8.9 (0–114.6)	7.8 (0–57.7)	8.0 (0–194.4)	8.6 (0–194.4)	7.1 (0–180.8)
GADA	45.6 (0.1–374.7)	11.4 (0.1–410.6) ∂	20.9 (0.1–410.6)	19.8 (0.1–374.7)	12.1 (0.1–374.7)	45.6 (0.1–410.6) #
IA-2A	2.6 (0.0–132.7)	15.9 (0.0–153.1)	0.7 (0.1–127.1)	14.6 (0.0–153.1)	16.9 (0.0–153.1)	2.6 (0.0–133.9)

* $P \leq 0.001$, † $P=0.03$, ‡ $P=0.002$, § $P=0.02$, || $P=0.045$, ¶ $P=0.007$, # $P=0.01$, ∂ $P=0.003$.

Rapid progression to T1D is associated with high levels of diabetes-associated autoantibodies

In the whole DIPP cohort, the initial ICA level was a predictor of T1D already at relatively low ICA titers (Publication II, Fig. 1B). In the analyses of diabetes-free survival, the 5-year progression rate for subjects with an initial ICA titer <10 JDFU was 5.7%, while the corresponding values for those with moderate (ICA 10-19 JDFU) and high ICA (≥20 JDFU) levels were 31.8% and 61.2%, respectively (P<0.001 in all comparisons). The median ICA level was higher in the progressors already in the first ICA-positive samples (15 JDFU vs. 5 JDFU in non-progressors, P<0.001; Publication II). During the follow-up, the difference in ICA levels between progressors and non-progressors increased, reaching maximal median titers of 168 JDFUs in progressors and 5 JDFU in non-progressors (P<0.001). The 5-year cumulative disease risk assessed, starting from the time point at which the maximal ICA level was reached, was 2.3% ($CI_{95\%}$ 0.3-4.3%) for children with low ICA level, while the comparable values for those with moderate and high ICA titers were 11.7% ($CI_{95\%}$ 2.9-20.5%) and 76.5% ($CI_{95\%}$ 61.4-91.6%; P<0.001 between all groups), respectively.

Although higher ICA titers correlated with higher numbers of detectable autoantibodies at sampling (r_S=0.68, P<0.001), the association between ICA level and T1D remained significant after adjusting for the number of positive autoantibodies (r_S=0.10, P<0.001; unpublished data). Comparable categorized assessments of the diabetes-free survival regarding the three other autoantibodies were not done, mainly because of the ICA-based screening strategy used in the DIPP study, but the levels of all these autoantibodies were higher in progressors both at the seroconversion based on ICA and at the time of appearance of maximal autoantibody positivity in the whole DIPP cohort (Table 13). The levels of ICA, IAA, and GADA were higher in the persistently multipositive DIPP children at all observation time points (Publication IV, Table 2).

Table 13. Levels of diabetes-associated autoantibodies in 1173 ICA-positive DIPP children at ICA-based seroconversion and at the time of the maximal combination of positive autoantibodies.

Levels of DAAs	**Non-diabetic subjects**	**Progressors**
	Median RU (range)	
At ICA-based seroconversion		
ICA	5 (3–640)	15 (4–668)
IAA	0.3 (0–146.1)	9.9 (0–167.8)
GADA	0.1 (0–1154.5)	15.4 (0.1–316.7)
IA-2A	0.1 (0–247.3)	0.3 (0.1–221.7)
At maximal combination of positive DAAs		
ICA	5 (2–1280)	35 (4–1742)
IAA	0.3 (0–146.1)	12.0 (0–213.1)
GADA	0.2 (0–1154.5)	26.2 (0–1009.4)
IA-2A	0.1 (0–247.3)	8.1 (0.1–221.7)

For all comparisons between non-diabetic subjects and progressors P<0.001.

Positivity for IAA and/or multipositivity are two early markers associated with high risk for T1D

In addition to the ICA titer, IAA positivity (n=238/1173 ICA-positive children, 20.3%), and positivity for multiple autoantibodies (n=161, 13.7%) were associated with increased disease risk. The progression rate in ICA-positive DIPP children with IAA positivity present at the time of seroconversion for ICA was 76.6% ($CI_{95\%}$ 56.7%-96.4%), while it was 11.2% ($CI_{95\%}$ 6.8%-15.6%) in children being IAA-negative at that time point ($P<0.001$; unpublished data). Similarly, the progression rate of initially multipositive children was 71.6% ($CI_{95\%}$ 59.7%-83.5%), whereas the rate was 20.6% ($CI_{95\%}$ 10.6%-30.6%) in children initially positive for a single autoantibody ($P<0.001$; Fig. 4A and B; unpublished data). The correlation between initial IAA positivity and multipositivity was high (adjusted $r_S=0.48$, $P<0.001$), and the correlations between T1D and each of these factors remained significant after controlling for the other factor (adjusted $r_S=0.36$ for IAA and $r_S=0.30$ for multipositivity, $P<0.001$ for both; unpublished data).

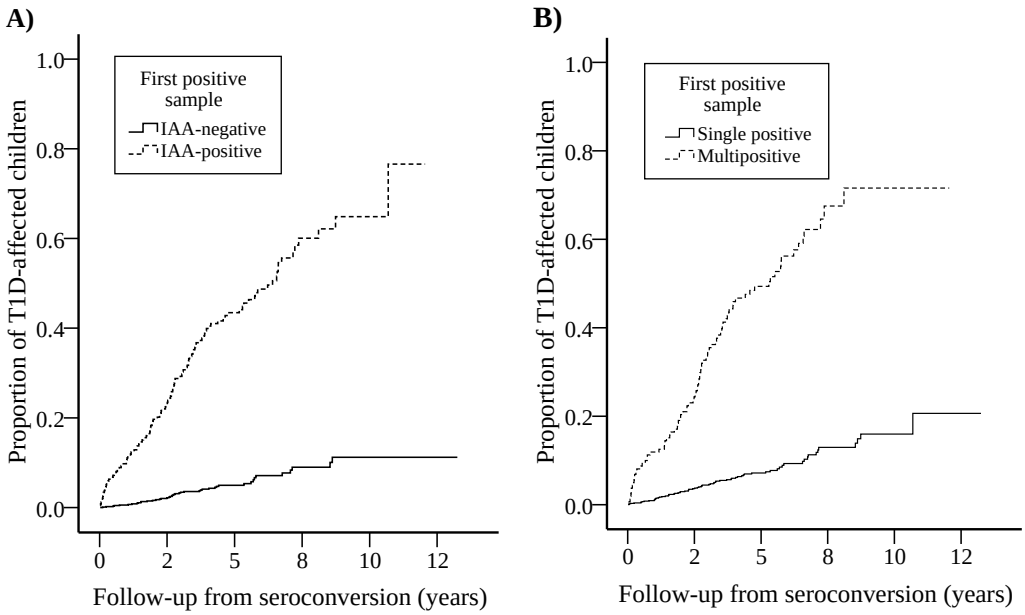

Figure 4. Progression to type 1 diabetes in 1173 ICA-positive DIPP children in relation to IAA positivity (**A**) and multipositivity (**B**) at seroconversion. $P<0.001$ for both.

Positivity for IAA at seroconversion was most common in DIPP children who had seroconverted under the age of 2 years, and the frequency of this phenomenon differed significantly between all groups categorized according to age at seroconversion (Table 11). On the other hand, the frequency of multipositivity appearing already at seroconversion was similar in all age groups except the oldest one, where multipositivity was rare (Table 11).

Persistence of positivity stratifies the T1D-associated risk of the diabetes-associated autoantibodies

Persistence of autoantibody positivity stratifies further the disease risk related to diabetes-associated autoantibodies. In the current DIPP cohort, the progression rate of children with transient ICA positivity was 1.6% ($CI_{95\%}$ 0-3.8%), whereas in children persistently positive for at least ICA the cumulative disease risk was 48.6% ($CI_{95\%}$ 35.2-62.1%; $P<0.001$; Fig. 5A). Persistent IAA positivity seemed to identify DIPP children with a high disease risk and rapid progression to T1D and differentiate the children from those with a lower disease risk and slower progression rate (Fig. 5B). The disease risk associated with persistent IAA positivity was 86.6% ($CI_{95\%}$ 77.1%-96.1%), while the risk was 34.1% ($CI_{95\%}$ 15.6%-52.5%) in ICA-positive subjects who lacked pIAA positivity (unpublished data).

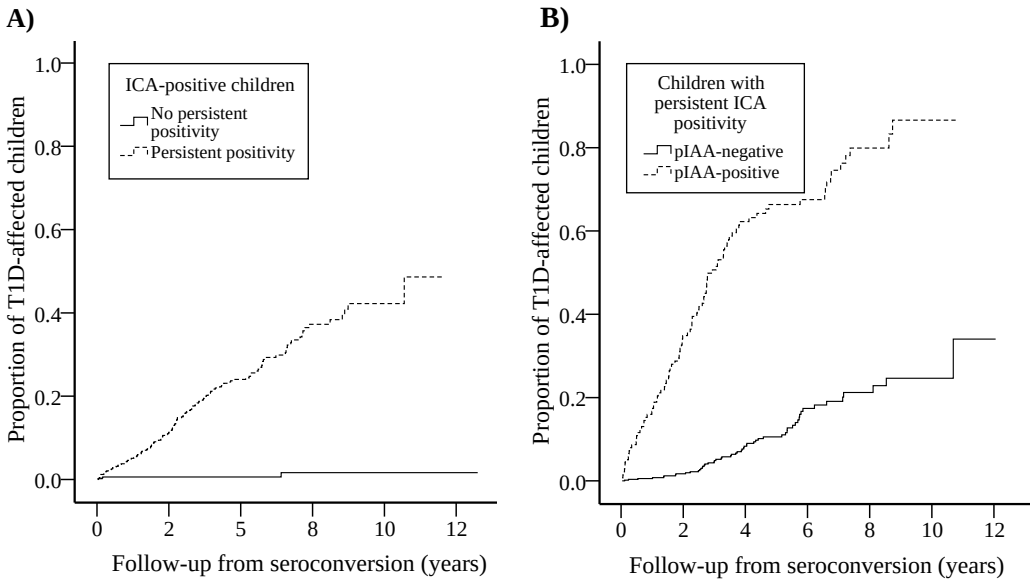

Figure 5. Progression to type 1 diabetes in ICA-positive DIPP children (**A**; n=1173) in relation to persistent ICA-based autoantibody positivity, and in persistently ICA-positive DIPP children (**B**; n=672) in relation to persistent IAA positivity. $P<0.001$ for both.

IAA affinity is high both in progressors and non-progressors among young IAA-positive DIPP children

As transient IAA positivity has been reported repeatedly to occur in young children, we assessed whether IAA affinity could be used to clarify the role of IAA positivity in the prediction of T1D (Publication III). IAA affinity was high in young IAA-positive DIPP children already at the appearance of IAA positivity, and the affinity level was similar in both progressors and children remaining unaffected or progressing to T1D at a slow pace (Publication III, Fig. 1–2). The affinity value remained at similar level in both groups of IAA-positive children, and no maturation of the immune response to insulin was

observed. IAA affinity was higher in multipositive samples, but the number of detectable autoantibodies did not correlate with IAA affinity (r_S=0.05, *P*=0.45; Publication III, Fig. 2A).

The predictive role of high vs. low antibody affinity in IAA-positive children (16 progressors among 29 children) was studied by choosing the median affinity value observed in the whole cohort ($5.0x10^9$ l/mol) as a cut-off value for binary categorization, and then analyzing the progression to multipositivity in children initially positive for IAA only (Publication III, Fig. 2A). The proportion of children progressing to multipositivity was similar in both groups regardless of IAA affinity. The same cut-off value was used when all participating IAA-positive children (n=128) were categorized by their initial IAA affinity values in order to analyze their progression to T1D. No differences were observed between the groups, and the finding remained the same even if lower cut-off values ($<1.0x10^9$ l/mol or $<3.0x10^9$ l/mol) were applied. Thus, the analysis of IAA affinity did not facilitate the assessment of the disease risk of young IAA-positive DIPP children.

The two groups of IAA-positive children were matched for the main background factors (gender, HLA genotype, geographic area, age at appearance of IAA, and availability of comparable IAA-positive samples). It became apparent that these groups had highly similar profiles for non-HLA gene polymorphisms and similar rates of participation in the intervention trial with intranasal insulin (Publication III, Table 2). INS variants or treatment with intranasal insulin appeared to have no effect on IAA level or affinity, and the change in IAA level observed in children in the insulin treatment group did not differ from that seen in children that did not participate in the intervention trial (*P*=0.45; Publication III, Table 3).

The protective PTPN22 CC variant was associated with slightly but significantly lower IAA levels than those observed in children carrying the high risk-associated TT variant (*P*=0.04). Increased IAA levels (Publication III, Fig. 1A) and the higher number of positive autoantibodies were the only findings differentiating young IAA-positive progressors from their more slowly progressing or non-progressing peers. At the first sampling, the levels of IAA were similar in both groups (medians 13.6 RU vs. 12.4 RU, *P*=0.23; Publication III, Fig. 1A), whereas at the second sampling the IAA level observed in progressors was higher than that seen in slowly progressing or non-progressing children (medians 20.1 RU vs. 11.1 RU, *P*=0.001). IAA levels did not correlate with the IAA affinity (r_S=0.11; *P*=0.08) or with the number of positive autoantibodies (r_s=0.09; *P*=0.19), although the frequency of multipositivity was higher in progressors at the initial seroconversion (59% vs. 38%; *P*=0.01), at the time of the first and the second IAA-positive samples (89% vs. 65%, *P*=0.001 and 98% vs. 82%, *P*=0.003), and at the end of the follow-up (98% vs. 83%, *P*=0.002; partly unpublished data).

Categorization of study subjects by their maximal autoantibody status leads to identification of profiles with highly variable disease risks

According to previous studies on unaffected family members of patients with T1D, the 5-year disease risk is approximately 50% among FDRs testing positive for multiple

diabetes-associated autoantibodies (Skyler et al. 2005). During the analyses for Publication II it became evident that the proportions of children progressing to T1D and the delay from the initial seroconversion to overt disease are highly variable in multipositive DIPP children. The correlation between disease progression and the number of positive autoantibodies at the time of ICA-based seroconversion was strong (r_S=0.65, P<0.001) in the whole cohort, but in the subjects who developed persistent multipositivity during the follow-up, the strength of the correlation between T1D and the maximal number of simultaneously positive autoantibodies was markedly lower (r_S=0.14), although the correlation was still significant (P=0.02, unpublished data).

To analyze the predictive role of ICA-based autoantibody combinations further, we categorized ICA-positive subjects by their maximal autoantibody status. To assess simultaneously the impact of the background factors, the risk estimates were calculated first for the whole cohort and follow-up time, and then for 5-year follow-up, family history of T1D, and for the HLA risk genotypes, separately (Table 14; partly unpublished data). Regarding sensitivity, the highest values of the ICA-based combinations were observed for positivity for all four autoantibodies (48%-58%), and in all, positivity for at least ICA was associated with a sensitivity ranging from 80% to 88% (Table 14). The sensitivity values for persistent triple and quadruple positivity were similar and differed only slightly from the values observed for persistent double positivity.

Disease specificity of all multipositive ICA-based combinations were high (98%-100%), and the highest of these values were associated with the combination of persistent ICA and IAA positivity (Table 14). The highest positive predictive values (PPVs; range 82%-100%) were observed for the combination of persistently positive ICA and IAA in all other subanalyses except for the high risk genotype, in which the combination with the highest PPV (100%) included also persistent IA-2A positivity (Table 14). Negative predictive values (NPVs) were high for all ICA-based combinations, and the highest ones (97%-99%) were seen for positivity for all four autoantibodies (Table 14).

Regarding the whole follow-up, the highest cumulative disease risk (100%) was associated with the combination of persistently positive ICA and IAA. This antibody combination represented also a risk marker for rapid prediabetic progression, since the cumulative disease risk was 82% already for the 5-year follow-up period. Among the double positive combinations, the combined ICA and GADA positivity resulted in low progression rates (0%-8%), thus lowering also the overall risk estimates of double positivity. In the current DIPP cohort, there were 15 progressors who were seronegative during the prediabetic follow-up time. Twelve of these 15 (80%) subjects did not attend the follow-up visits as scheduled by the DIPP study protocol, and the median delay from the last visit to diagnosis was 3.8 years (range 1.9-6.2 years). All the prediabetically seronegative progressors who had an autoantibody sample available at diagnosis were seropositive at that time, and all but one had developed positivity for multiple autoantibodies. Measurement of the ZnT8A might have revealed beta-cell autoimmunity in those three DIPP children who tested seronegative only some months before their diagnosis, but unfortunately that data was unavailable.

Table 14A-E. Summary on the predictive characteristics (sensitivity [**A**], specificity [**B**], positive predictive value [PPV; **C**], negative predictive value [NPV; **D**], and cumulative disease risk [**E**]) of the four diabetes-associated autoantibodies for type 1 diabetes during the whole follow-up and for the first five years after reaching the maximal autoantibody status, and in relation to family history of T1D and HLA genotypes. *Categorization is based on ICA positivity. In the case of defined autoantibody categories, each individual is included in only one category. Boldface numbers represent the highest values observed for the combinations of autoantibodies.

A

SENSITIVITY % ($CI_{95\%}$)						
Combinations of autoantibodies	**Whole follow-up**	**5-year follow-up**	**Non-FDRs**	**FDRs**	**High risk**	**Moderate risk**
ICA the only positive DAA	0.6 (0–3.1)	2.0 (0.4–5.8)	0.6 (0–3.5)	0 (0–14.2)	0 (0–4.5)	1.0 (0–5.4)
Double positivity*	7.8 (4.3–12.7)	8.2 (4.3–13.8)	7.7 (4.0–13.1)	8.3 (1–27)	10.0 (4.4–18.8)	6.0 (2.2–12.6)
ICA, IAA	5.6 (2.7–10.0)	6.1 (2.8–11.3)	5.1 (2.2–9.9)	8.3 (1–27)	7.5 (2.8–15.6)	4.0 (1.1–9.9)
ICA, GADA	1.1 (0.1–4.0)	0 (0–2.5)	1.3 (0.2–4.6)	0 (0–14.2)	1.3 (0–6.8)	1.0 (0–5.4)
ICA, IA-2A	1.1 (0.1–4.0)	2.0 (0.4–5.8)	1.3 (0.2–4.6)	0 (0–14.2)	1.3 (0–6.8)	1.0 (0–5.4)
Triple positivity*	23.3 (17.4–30.2)	21.8 (15.4–29.3)	21.8 (15.6–29.1)	33.3 (15.6–55.3)	27.5 (18.1–38.6)	20.0 (12.7–29.2)
ICA, IAA, GADA	8.3 (4.7–13.4)	8.2 (4.3–13.8)	8.3 (4.5–13.8)	8.3 (1–27)	15.0 (8.0–24.7)	3.0 (0.6–8.5)
ICA, IAA, IA-2A	11.1 (6.9–16.6)	8.8 (4.8–14.6)	10.3 (6.0–16.1)	16.7 (4.7–37.4)	10.0 (4.4–18.8)	12.0 (6.4–20.0)
ICA, GADA, IA-2A	3.9 (1.6–7.8)	4.8 (1.9–9.6)	3.2 (1.0–7.3)	8.3 (1–27)	2.5 (0.3–8.7)	5.0 (1.6–11.3)
All four DAA positive	**54.4 (46.9–61.9)**	**48.3 (40.0–56.7)**	**55.8 (47.6–63.7)**	**45.8 (25.6–67.2)**	**50.0 (38.6–61.4)**	**58.0 (47.7–67.8)**
Positive for at least ICA	86.1 (80.2–90.8)	80.3 (72.9–86.4)	85.9 (79.4–90.9)	87.5 (67.6–97.3)	87.5 (78.2–93.8)	85.0 (76.5–91.4)
≥ 2 positive DAA*	85.6 (79.6–90.3)	78.2 (70.7–84.6)	85.3 (78.7–90.4)	87.5 (67.6–97.3)	87.5 (78.2–93.8)	84.0 (75.3–90.6)
≥ 3 positive DAA*	77.8 (71.0–83.6)	70.1 (62.0–77.3)	77.6 (70.2–83.8)	79.2 (57.8–92.9)	77.5 (66.8–86.1)	78.0 (68.6–85.7)
Only ICA persistently positive	3.3 (1.2–7.1)	4.1 (1.5–8.7)	3.2 (1.0–7.3)	4.2 (0.1–21.1)	2.5 (0.3–8.7)	4.0 (1.1–9.9)
Persistent double positivity*	20.6 (14.9–27.2)	17.7 (11.9–24.8)	21.2 (15.0–28.4)	16.7 (4.7–37.4)	17.5 (9.9–27.6)	23.0 (15.2–32.5)
pICA, pIAA	6.1 (3.1–10.7)	6.1 (2.8–11.3)	5.8 (2.7–10.7)	8.3 (1–27)	7.5 (2.8–15.6)	5.0 (1.6–11.3)
pICA, pGADA	1.1 (0.1–4.0)	1.4 (0.2–4.8)	0.6 (0–3.5)	4.2 (0.1–21.1)	2.5 (0.3–8.7)	0 (0.0–3.6)
pICA, pIA-2A	13.3 (8.7–19.2)	10.2 (5.8–16.3)	14.7 (9.6–21.3)	4.2 (0.1–21.1)	7.5 (2.8–15.6)	18.0 (11.0–26.9)
Persistent triple positivity*	29.4 (22.9–36.7)	25.2 (18.4–33)	29.5 (22.5–37.3)	29.2 (12.6–51.1)	**33.8 (23.6–45.2)**	26.0 (17.7–35.7)
pICA, pIAA, pGADA	5.6 (2.7–10.0)	6.1 (2.8–11.3)	5.8 (2.7–10.7)	4.2 (0.1–21.1)	11.3 (5.3–20.3)	1.0 (0–5.4)
pICA, pIAA, pIA-2A	11.1 (6.9–16.6)	10.9 (6.4–17.1)	10.9 (6.5–16.9)	12.5 (2.7–32.4)	11.3 (5.3–20.3)	11.0 (5.6–18.8)
pICA, pGADA, pIA-2A	12.8 (8.3–18.6)	8.2 (4.3–13.8)	12.8 (8.0–19.1)	12.5 (2.7–32.4)	11.3 (5.3–20.3)	14.0 (7.9–22.4)
All four DAA persistently positive	**30.6 (23.9–37.8)**	**27.9 (20.8–35.9)**	**30.1 (23.1–38.0)**	**33.3 (15.6–55.3)**	31.3 (21.3–42.6)	**30.0 (21.2–40.0)**
Persistently positive for at least ICA	83.9 (77.7–88.9)	74.8 (67.0–81.6)	84.0 (77.3–89.4)	83.3 (62.6–95.3)	85.0 (75.3–92.0)	83.0 (74.2–89.8)
≥ 2 DAA persistently positive*	80.6 (74.0–86.1)	70.7 (62.7–78.0)	80.8 (73.7–86.6)	79.2 (57.8–92.9)	82.5 (72.4–90.1)	79.0 (69.7–86.5)
≥ 3 DAA persistently positive*	60.0 (52.4–67.2)	53.1 (44.7–61.3)	59.6 (51.5–67.4)	62.5 (40.6–81.2)	65.0 (53.5–75.3)	56.0 (45.7–65.9)

*Categorization is based on ICA positivity. In the case of defined autoantibody categories, each individual is included in only one category.

B

SPECIFICITY % ($CI_{95\%}$) Combinations of autoantibodies	Whole follow-up	5-year follow-up	Non-FDRs	FDRs	High risk	Moderate risk
ICA the only positive DAA	89.2 (88.5–89.9)	96.7 (96.2–97.1)	89.1 (88.4–89.8)	92.8 (87.5–96.4)	87.7 (85.9–89.3)	89.6 (88.8–90.4)
Double positivity*	98.4 (98.1–98.7)	99.7 (99.5–99.8)	98.5 (98.1–98.7)	96.7 (92.5–98.9)	97.7 (96.8–98.4)	98.6 (98.3–98.9)
ICA, IAA	99.4 (99.2–99.5)	99.9 (99.8–99.9)	99.4 (99.2–99.5)	98.7 (95.4–99.8)	99.2 (98.6–99.6)	99.4 (99.2–99.6)
ICA, GADA	99.2 (99.0–99.4)	99.8 (99.7–99.9)	99.2 (99.0–99.4)	98.7 (95.4–99.8)	98.7 (97.9–99.2)	99.3 (99.1–99.5)
ICA, IA-2A	99.9 (99.7–99.9)	**100 (99.9–100)**	99.9 (99.8–99.9)	**99.3 (96.4–100)**	99.9 (99.5–100)	99.9 (99.7–99.9)
Triple positivity*	99.4 (99.2–99.5)	99.7 (99.5–99.8)	99.5 (99.3–99.6)	94.8 (90–97.7)	98.9 (98.2–99.3)	99.5 (99.3–99.7)
ICA, IAA, GADA	99.7 (99.6–99.8)	99.8 (99.7–99.9)	99.8 (99.6–99.9)	96.7 (92.5–98.9)	99.5 (98.9–99.8)	99.8 (99.6–99.9)
ICA, IAA, IA-2A	**99.9 (99.8–100)**	99.9 (99.8–100)	**99.9 (99.8–100)**	**99.3 (96.4–100)**	**100 (99.8–100)**	**99.9 (99.7–100)**
ICA, GADA, IA-2A	99.8 (99.6–99.9)	**100 (99.9–100)**	99.8 (99.7–99.9)	98.7 (95.4–99.8)	99.4 (98.9–99.7)	99.9 (99.7–99.9)
All four DAA positive	98.9 (98.7–99.1)	99.3 (99.0–99.5)	99.0 (98.7–99.2)	97.4 (93.4–99.3)	98.7 (97.9–99.2)	99.0 (98.7–99.2)
Positive for at least ICA	85.9 (85.1–86.7)	95.3 (94.8–95.8)	86.0 (85.2–86.8)	81.7 (74.6–87.5)	84.6 (82.7–86.5)	86.7 (85.8–87.6)
≥ 2 positive DAA*	96.7 (96.3–97.1)	98.6 (98.3–98.9)	96.9 (96.5–97.3)	88.9 (82.8–93.4)	95.3 (94.0–96.3)	97.1 (96.6–97.5)
≥ 3 positive DAA*	98.3 (98.0–98.6)	99.0 (98.7–99.2)	98.4 (98.1–98.7)	92.2 (86.7–95.9)	97.5 (96.6–98.3)	98.5 (98.2–98.8)
Only ICA persistently positive	94.7 (94.2–95.2)	98.2 (97.8–98.5)	94.7 (94.2–95.2)	94.8 (90–97.7)	94.4 (93.2–95.6)	94.8 (94.2–95.3)
Persistent double positivity*	99.4 (99.2–99.5)	99.8 (99.6–99.9)	99.4 (99.2–99.6)	96.7 (92.5–98.9)	99.2 (98.6–99.6)	99.4 (99.2–99.6)
pICA, pIAA	**100 (99.9–100)**	**100 (99.9–100)**	**100 (99.9–100)**	**100 (97.6–100)**	99.9 (99.6–100)	**100 (99.9–100)**
pICA, pGADA	99.7 (99.5–99.8)	99.9 (99.8–100)	99.7 (99.5–99.8)	98.7 (95.4–99.8)	99.4 (98.9–99.7)	99.7 (99.6–99.9)
pICA, pIA-2A	99.7 (99.6–99.8)	99.9 (99.7–99.9)	99.8 (99.6–99.9)	98 (94.4–99.6)	99.9 (99.5–100)	99.7 (99.5–99.8)
Persistent triple positivity*	99.1 (98.8–99.3)	99.6 (99.4–99.7)	99.1 (98.9–99.4)	96.1 (91.7–98.5)	98.3 (97.5–98.9)	99.3 (99.0–99.5)
pICA, pIAA, pGADA	99.9 (99.8–100)	99.9 (99.8–100)	99.9 (99.8–100)	98 (94.4–99.6)	99.7 (99.3–99.9)	99.9 (99.8–100)
pICA, pIAA, pIA-2A	99.9 (99.8–100)	99.9 (99.8–99.9)	99.9 (99.8–100)	99.3 (96.4–100)	**100 (99.8–100)**	99.9 (99.7–99.9)
pICA, pGADA, pIA-2A	99.3 (99.1–99.5)	99.8 (99.7–99.9)	99.3 (99.1–99.5)	98.7 (95.4–99.8)	98.6 (97.9–99.1)	99.5 (99.2–99.6)
All four DAA persistently positive	99.7 (99.5–99.8)	99.6 (99.4–99.7)	99.7 (99.5–99.8)	99.3 (96.4–100)	99.6 (99.1–99.9)	99.7 (99.5–99.8)
Persistently positive for at least ICA	92.8 (92.2–93.4)	97.1 (96.7–97.5)	92.9 (92.3–93.5)	86.9 (80.5–91.8)	91.6 (90.0–92.9)	93.1 (92.4–93.8)
≥ 2 DAA persistently positive*	98.1 (97.7–98.4)	99.0 (98.7–99.2)	98.2 (97.9–98.5)	92.2 (86.7–95.9)	97.1 (96.1–97.9)	98.3 (98.0–98.7)
≥ 3 DAA persistently positive*	98.7 (98.4–99.0)	99.2 (99.0–99.4)	98.8 (98.5–99.0)	95.4 (90.8–98.1)	97.9 (97.1–98.6)	98.9 (98.6–99.2)

*Categorization is based on ICA positivity. In the case of defined autoantibody categories, each individual is included in only one category.

C

POSITIVE PREDICTIVE VALUE % ($CI_{95\%}$)						
Combinations of autoantibodies	**Whole follow-up**	**5-year follow-up**	**Non-FDRs**	**FDRs**	**High risk**	**Moderate risk**
ICA the only positive DAA	0.1 (0–0.7)	1.4 (0.3–3.9)	0.1 (0.0–0.7)	0 (0–28.5)	0 (0–2.0)	0.2 (0.0–0.9)
Double positivity*	10.9 (6.1–17.7)	36.4 (20.4–54.9)	9.9 (5.2–16.7)	28.6 (3.7–71)	19.0 (8.6–34.1)	7.0 (2.6–14.6)
ICA, IAA	17.9 (8.9–30.4)	52.9 (27.8–77.0)	15.4 (6.9–28.1)	50 (6.8–93.2)	33.3 (13.3–59.0)	10.5 (2.9–24.8)
ICA, GADA	3.3 (0.4–11.5)	0 (0–30.8)	3.4 (0.4–11.9)	0 (0–84.2)	4.8 (0.1–23.8)	2.6 (0.1–13.5)
ICA, IA-2A	16.7 (2.1–48.4)	50.0 (11.8–88.2)	18.2 (2.3–51.8)	0 (0–97.5)	33.3 (0.8–90.6)	11.1 (0.3–48.2)
Triple positivity*	48.3 (37.4–59.2)	61.5 (47.0–74.7)	47.9 (35.9–60.1)	50 (24.7–75.3)	56.4 (39.6–72.2)	41.7 (27.6–56.8)
ICA, IAA, GADA	41.7 (25.5–59.2)	54.5 (32.2–75.6)	44.8 (26.4–64.3)	28.6 (3.7–71)	60.0 (36.1–80.9)	18.8 (4.0–45.6)
ICA, IAA, IA-2A	**74.1 (53.7–88.9)**	65.0 (40.8–84.6)	**72.7 (49.8–89.3)**	**80 (28.4–99.5)**	**100 (63.1–100)**	**63.2 (38.4–83.7)**
ICA, GADA, IA-2A	29.2 (12.6–51.1)	**70.0 (34.8–93.3)**	25.0 (8.7–49.1)	50 (6.8–93.2)	18.2 (2.3–51.8)	38.5 (13.9–68.4)
All four DAA positive	55.7 (48.0–63.2)	59.2 (49.8–68.0)	54.0 (46.0–61.9)	73.3 (44.9–92.2)	66.7 (53.3–78.3)	50.0 (40.6–59.4)
Positive for at least ICA	13.2 (11.3–15.3)	27.6 (23.4–32.1)	11.9 (10.1–14.0)	42.9 (28.8–57.8)	23.7 (19.0–29.0)	10.0 (8.1–12.2)
≥ 2 positive DAA*	39.4 (34.5–44.4)	56.1 (49.0–63.0)	37.7 (32.6–43.0)	55.3 (38.3–71.4)	49.6 (41.1–58.2)	33.6 (27.8–39.8)
≥ 3 positive DAA*	53.2 (47.0–59.4)	59.9 (52.1–67.3)	52.2 (45.5–58.7)	61.3 (42.2–78.2)	62.6 (52.3–72.1)	47.6 (39.7–55.5)
Only ICA persistently positive	1.5 (0.6–3.3)	4.7 (1.8–10.0)	1.3 (0.4–3.0)	11.1 (0.3–48.2)	2.4 (0.3–8.2)	1.3 (0.4–3.3)
Persistent double positivity*	45.1 (34.1–56.5)	61.9 (45.6–76.4)	45.2 (33.5–57.3)	44.4 (13.7–78.8)	53.8 (33.4–73.4)	41.1 (28.1–55.0)
pICA, pIAA	91.7 (61.5–99.8)	**81.8 (48.2–97.7)**	**90.0 (55.5–99.7)**	**100 (15.8–100)**	85.7 (42.1–99.6)	**100 (47.8–100)**
pICA, pGADA	7.7 (0.9–25.1)	28.6 (3.7–71.0)	4.3 (0.1–21.9)	33.3 (0.8–90.6)	18.2 (2.3–51.8)	0 (0–21.8)
pICA, pIA-2A	54.5 (38.8–69.9)	62.5 (40.6–81.2)	57.5 (40.9–73.0)	25 (0.6–80.6)	75.0 (34.9–96.8)	50.0 (32.9–67.1)
Persistent triple positivity*	43.8 (34.8–53.1)	58.7 (45.6–71.0)	42.6 (33.1–52.5)	53.8 (25.1–80.8)	51.9 (37.6–66.0)	37.7 (26.3–50.2)
pICA, pIAA, pGADA	55.6 (30.8–78.5)	60.0 (32.3–83.7)	64.3 (35.1–87.2)	25 (0.6–80.6)	69.2 (38.6–90.9)	20.0 (0.5–71.6)
pICA, pIAA, pIA-2A	**71.4 (51.3–86.8)**	66.7 (44.7–84.4)	70.8 (48.9–87.4)	75 (19.4–99.4)	**100 (66.4–100)**	57.9 (33.5–79.7)
pICA, pGADA, pIA-2A	30.7 (20.5–42.4)	50.0 (29.1–70.9)	28.6 (18.4–40.6)	60 (14.7–94.7)	30.0 (14.7–49.4)	31.1 (18.2–46.6)
All four DAA persistently positive	68.8 (57.4–78.7)	60.3 (47.7–72.0)	66.2 (54.0–77.0)	88.9 (51.8–99.7)	80.6 (62.5–92.5)	61.2 (46.2–74.8)
Persistently positive for at least ICA	22.5 (19.4–25.8)	36.7 (31.2–42.4)	20.7 (17.6–24.1)	50 (33.8–66.2)	35.1 (28.4–42.2)	17.4 (14.1–21.1)
≥ 2 DAA persistently positive*	51.2 (45.3–57.2)	60.1 (52.4–67.5)	50.0 (43.7–56.3)	61.3 (42.2–78.2)	60.6 (50.7–69.8)	45.4 (37.9–53.1)
≥ 3 DAA persistently positive*	53.7 (46.6–60.8)	59.5 (50.6–68.0)	52.0 (44.4–59.5)	68.2 (45.1–86.1)	62.7 (51.3–73.0)	47.5 (38.2–56.9)

*Categorization is based on ICA positivity. In the case of defined autoantibody categories, each individual is included in only one category.

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NEGATIVE PREDICTIVE VALUE % ($CI_{95\%}$)						
Combinations of autoantibodies	**Whole follow-up**	**5-year follow-up**	**Non-FDRs**	**FDRs**	**High risk**	**Moderate risk**
ICA the only positive DAA	97.3 (96.9–97.7)	97.8 (97.4–98.1)	97.6 (97.2–98.0)	85.5 (79.3–90.5)	94.2 (92.9–95.4)	98.1 (97.7–98.5)
Double positivity*	97.7 (97.4–98.1)	98.0 (97.6–98.3)	98.0 (97.6–98.3)	87.1 (81.1–91.7)	95.3 (94.1–96.3)	98.4 (98.0–98.7)
ICA, IAA	97.7 (97.3–98.0)	97.9 (97.6–98.3)	97.9 (97.6–98.3)	87.3 (81.4–91.9)	95.2 (94.1–96.2)	98.3 (98.0–98.7)
ICA, GADA	97.6 (97.2–97.9)	97.8 (97.4–98.2)	97.9 (97.5–98.2)	86.3 (80.3–91)	94.9 (93.7–96.0)	98.3 (97.9–98.6)
ICA, IA-2A	97.6 (97.2–97.9)	97.9 (97.5–98.2)	97.9 (97.5–98.2)	86.4 (80.4–91.1)	95.0 (93.8–96.0)	98.3 (97.9–98.6)
Triple positivity*	98.1 (97.8–98.4)	98.3 (97.9–98.6)	98.3 (98.0–98.6)	90.1 (84.4–94.2)	96.2 (95.1–97.1)	98.6 (98.3–98.9)
ICA, IAA, GADA	97.8 (97.4–98.1)	98.0 (97.6–98.3)	98.0 (97.7–98.3)	87.1 (81.1–91.7)	95.6 (94.5–96.6)	98.3 (98.0–98.6)
ICA, IAA, IA-2A	97.8 (97.5–98.2)	98.0 (97.6–98.3)	98.1 (97.7–98.4)	88.4 (82.6–92.8)	95.4 (94.2–96.4)	98.5 (98.1–98.8)
ICA, GADA, IA-2A	97.7 (97.3–98.0)	97.9 (97.6–98.2)	97.9 (97.5–98.2)	87.3 (81.4–91.9)	95.0 (93.8–96.0)	98.4 (98.0–98.7)
All four DAA positive	**98.9 (98.6–99.1)**	**98.9 (98.6–99.1)**	**99.0 (98.8–99.2)**	**92 (86.7–95.7)**	**97.4 (96.4–98.1)**	**99.3 (99.0–99.5)**
Positive for at least ICA	99.6 (99.4–99.7)	99.5 (99.3–99.7)	99.6 (99.5–99.8)	97.7 (93.3–99.5)	99.2 (98.5–99.6)	99.7 (99.5–99.8)
≥ 2 positive DAA*	99.6 (99.5–99.8)	99.5 (99.3–99.7)	99.7 (99.5–99.8)	97.8 (93.8–99.6)	99.3 (98.7–99.7)	99.7 (99.5–99.8)
≥ 3 positive DAA*	99.4 (99.2–99.6)	99.3 (99.1–99.5)	99.5 (99.3–99.7)	96.6 (92.2–98.9)	98.8 (98.1–99.3)	99.6 (99.4–99.8)
Only ICA persistently positive	97.5 (97.1–97.9)	97.9 (97.5–98.2)	97.8 (97.4–98.1)	86.3 (80.2–91.1)	94.8 (93.5–95.8)	98.3 (97.9–98.6)
Persistent double positivity*	98.0 (97.7–98.4)	98.2 (97.8–98.5)	98.3 (98.0–98.6)	88.1 (82.2–92.6)	95.7 (94.6–96.7)	98.7 (98.3–98.9)
pICA, pIAA	97.7 (97.3–98.0)	98.0 (97.6–98.3)	98.0 (97.6–98.3)	87.4 (81.6–92)	95.3 (94.1–96.3)	98.4 (98.0–98.7)
pICA, pGADA	97.6 (97.2–97.9)	97.8 (97.5–98.2)	97.9 (97.5–98.2)	86.8 (80.8–91.4)	95.0 (93.8–96.0)	98.3 (97.9–98.6)
pICA, pIA-2A	97.9 (97.5–98.2)	98.0 (97.7–98.4)	98.2 (97.8–98.4)	86.7 (80.7–91.4)	95.3 (94.1–96.3)	98.6 (98.2–98.9)
Persistent triple positivity*	98.3 (97.9–98.5)	98.4 (98.0–98.6)	98.5 (98.1–98.7)	89.6 (83.9–93.8)	**96.5 (95.5–97.4)**	98.7 (98.4–99.0)
pICA, pIAA, pGADA	97.7 (97.3–98.0)	98.0 (97.6–98.3)	98.0 (97.6–98.3)	86.7 (80.7–91.4)	95.5 (94.3–96.4)	98.3 (97.9–98.6)
pICA, pIAA, pIA-2A	97.8 (97.5–98.2)	98.1 (97.7–98.4)	98.1 (97.7–98.4)	87.9 (82–92.3)	95.5 (94.3–96.4)	98.5 (98.1–98.8)
pICA, pGADA, pIA-2A	97.9 (97.5–98.2)	98.0 (97.6–98.3)	98.1 (97.8–98.4)	87.8 (81.9–92.3)	95.4 (94.2–96.4)	98.5 (98.2–98.8)
All four DAA persistently positive	**98.3 (98.0–98.6)**	**98.4 (98.1–98.7)**	**98.5 (98.2–98.7)**	**90.5 (85–94.5)**	96.4 (95.4–97.3)	**98.8 (98.5–99.1)**
Persistently positive for at least ICA	99.6 (99.4–99.7)	99.4 (99.2–99.6)	99.6 (99.4–99.8)	97.1 (92.7–99.2)	99.1 (98.5–99.6)	99.7 (99.5–99.8)
≥ 2 DAA persistently positive*	99.5 (99.3–99.7)	99.3 (99.1–99.5)	99.6 (99.4–99.7)	96.6 (92.2–98.9)	99.0 (98.4–99.5)	99.6 (99.4–99.8)
≥ 3 DAA persistently positive*	99.0 (98.7–99.2)	99.0 (98.7–99.2)	99.1 (98.9–99.3)	94.2 (89.3–97.3)	98.1 (97.3–98.7)	99.2 (99.0–99.4)

*Categorization is based on ICA positivity. In the case of defined autoantibody categories, each individual is included in only one category.

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CUMULATIVE DISEASE RISK % ($CI_{95\%}$)						
Combinations of autoantibodies	**Whole follow-up**	**5-year follow-up**	**Non-FDRs**	**FDRs**	**High risk**	**Moderate risk**
ICA the only positive DAA	0.3 (0–0.8)	1.4 (0–2.9)	0.3 (0–0.8)	0 (0–0)	0 (0–0)	0.4 (0–1.1)
Double positivity*	16.0 (7.4–24.6)	36.4 (20.0–52.8)	12.3 (5.6–19.1)	35.7 (0–77.0)	25.4 (9.4–41.4)	11.3 (1.6–21.1)
ICA, IAA	24.9 (9.8–40.0)	52.9 (29.2–76.7)	18.4 (6.3–30.5)	62.5 (6.4–100)	39.4 (13.2–65.6)	17.8 (0–36.0)
ICA, GADA	4.4 (0–10.5)	0 (0–0)	4.2 (0–9.9)	0 (0–0)	8.3 (0–24.0)	2.6 (0–7.5)
ICA, IA-2A	25.0 (0–55.0)	50.0 (10.0–90.0)	25.0 (0–55.0)	0 (0–0)	50.0 (0–100)	16.7 (0–46.5)
Triple positivity*	66.7 (52.8–80.7)	61.5 (48.3–74.8)	63.2 (48.6–77.8)	67.9 (36.6–99.3)	74.0 (55.4–92.6)	57.2 (39.1–75.4)
ICA, IAA, GADA	54.2 (33.3–75.1)	54.5 (33.7–75.4)	51.7 (31.6–71.8)	42.9 (0–91.7)	72.0 (48.5–95.5)	24.2 (0–48.7)
ICA, IAA, IA-2A	**84.7 (69.2–100)**	65.0 (44.1–85.9)	**82.1 (64.2–100)**	**100 (100–100)**	**100 (100–100)**	**75.4 (52.5–98.4)**
ICA, GADA, IA-2A	41.5 (17.4–65.5)	**70.0 (41.6–98.4)**	45.5 (13.8–77.2)	66.7 (13.3–100)	25.0 (0–55.0)	53.8 (21.1–86.6)
All four DAA positive	76.5 (66.0–87.1)	59.2 (50.4–68.0)	77.9 (62.7–93.0)	88.9 (68.9–100)	85.5 (72.0–99.0)	72.5 (57.9–87.1)
Positive for at least ICA	24.7 (20.1–29.3)	27.6 (23.4–31.9)	27.2 (18.2–36.2)	67.1 (47.1–87.2)	35.4 (27.0–43.8)	19.8 (14.4–25.1)
≥ 2 positive DAA*	63.1 (54.2–72.0)	56.1 (49.3–62.9)	64.3 (47.2–81.4)	77.0 (57.4–96.6)	74.7 (62.4–87.1)	56.5 (44.3–68.6)
≥ 3 positive DAA*	73.5 (64.9–82.1)	59.9 (52.6–67.2)	76.2 (61.1–91.3)	82.4 (63.0–100)	81.3 (70.1–92.5)	69.0 (56.6–81.4)
Only ICA persistently positive	2.9 (0.5–5.3)	4.7 (1.0–8.4)	2.5 (0.2–4.8)	16.7 (0–46.5)	3.4 (0–8.0)	2.8 (0–5.5)
Persistent double positivity*	76.4 (59.5–93.3)	61.9 (47.2–76.6)	67.1 (51.3–83.0)	46.7 (12.7–80.6)	**100 (100–100)**	68.8 (48.3–89.3)
pICA, pIAA	**100 (100–100)**	**81.8 (59.0–100)**	**100 (100–100)**	**100 (100–100)**	**100 (100–100)**	**100 (100–100)**
pICA, pGADA	11.2 (0.0–25.8)	28.6 (0–62.0)	6.3 (0–18.1)	33.3 (0–86.7)	23.8 (0–52.8)	0 (0–0)
pICA, pIA-2A	77.7 (60.3–95.0)	62.5 (43.1–81.9)	79.4 (61.2–97.5)	25.0 (0–67.4)	**100 (100–100)**	70.6 (49.7–91.4)
Persistent triple positivity*	64.8 (49.5–80.1)	58.7 (46.6–70.9)	70.9 (46.0–95.9)	72.3 (42.1–100)	65.5 (47.3–83.7)	67.8 (43.1–92.5)
pICA, pIAA, pGADA	72.8 (47.4–98.2)	60.0 (35.2–84.8)	87.8 (65.6–100)	25.0 (0–67.4)	20.0 (0–55.1)	20.0 (0–55.1)
pICA, pIAA, pIA-2A	87.0 (66.9–100)	66.7 (47.8–85.5)	83.6 (59.0–100)	100 (100–100)	63.3 (39.6–87.1)	63.3 (39.6–87.1)
pICA, pGADA, pIA-2A	52.6 (32.6–72.5)	50.0 (30.0–70.0)	61.2 (28.2–94.3)	100 (100–100)	63.5 (35.1–91.9)	63.5 (35.1–91.9)
All four DAA persistently positive	83.4 (71.7–95.0)	60.3 (48.7–71.9)	81.4 (67.2–95.5)	88.9 (68.4–100)	94.7 (84.7–100)	71.2 (55.0–87.4)
Persistently positive for at least ICA	41.2 (34.1–48.3)	36.7 (31.2–42.1)	45.9 (31.8–60.0)	71.3 (51.1–91.4)	53.8 (42.7–64.8)	34.6 (25.8–43.5)
≥ 2 DAA persistently positive*	73.2 (64.5–81.8)	60.1 (52.8–67.4)	75.5 (60.2–90.8)	81.6 (61.7–100)	82.4 (71.1–93.8)	67.5 (55.4–79.7)
≥ 3 DAA persistently positive*	73.0 (63.0–83.0)	59.5 (51.1–67.9)	75.6 (59.7–91.5)	89.0 (70.5–100)	80.5 (68.0–93.0)	80.5 (68.0–93.0)

*Categorization is based on ICA positivity. In the case of defined autoantibody categories, each individual is included in only one category.

To summarize the effects and to assess the proportional hazards of the baseline factors (gender, HLA genotype, and family history with T1D), age at seroconversion, and autoantibody data at ICA-based seroconversion (ICA, IAA, GADA, and IA-2A positivity and levels, and the number of positive autoantibodies) in the whole DIPP study cohort, a Cox regression analysis was performed. According to this analysis, the highest hazard ratio (HR) was associated with IAA positivity in the first ICA-positive sample (HR=3.1, $CI_{95\%}$ 1.7-5.8; $P<0.001$), followed by the number of positive autoantibodies at seroconversion (HR=1.9, $CI_{95\%}$ 1.5-2.4; $P<0.001$), positive family history for T1D (HR=1.8, $CI_{95\%}$ 1.1-3.0; $P=0.02$), high-risk HLA genotype (HR=1.6, $CI_{95\%}$ 1.2-2.3; $P=0.003$), IAA level in the first ICA-positive sample (HR=1.010, $CI_{95\%}$ 1.003-1.017; $P=0.003$), and ICA level in the first ICA-positive sample (HR=1.004, $CI_{95\%}$ 1.002-1.006; $P<0.001$; Unpublished data).

Predictive values of ICA-based autoantibody combinations are markedly similar in comparable age groups of children recruited from the general population and among family members of patients with T1D

Pairwise comparison of results of the subanalyses (whole follow-up time vs. 5-year follow-up, positive vs. negative family history of T1D, and high-risk vs. moderate-risk HLA genotypes; Table 14) showed that the maximal differences in the estimates of sensitivity, specificity, and NPV obtained from the subgroups varied from 9% to 12%, while the maximal differences regarding the estimates of PPV and cumulative risk were markedly higher, 49% and 63%, respectively. In general, higher sensitivity and cumulative disease risk values were associated with high-risk HLA genotype, positive family history with T1D and longer follow-up period, while the opposite was true for specificity and NPV. The variation observed for PPV was high, but to generalize, higher PPV was associated with shorter follow-up time, high-risk genotype and positive family history for T1D (partly unpublished data).

The predictive role of GADA and IA-2A positivity was assessed in a direct comparison between two cohorts of Finnish children of comparable ages (Publication I). According to the findings of that study, both GADA, IA-2A, and combined positivity resulted in statistically similar sensitivity values in the comparisons between siblings of affected children and children originating from the general population (68% vs. 50% for GADA; 58% vs. 43% for IA-2A; 48% vs. 36% for combined positivity, respectively, $P>0.05$ for all comparisons). Cumulative disease risks of single GADA and IA-2A positivity were higher in siblings of affected children than in the general population-based cohort (61% vs. 24%, respectively, [$P<0.001$] for GADA and 74%; vs. 32%, respectively [$P=0.002$] for IA-2A), but combined positivity indicated similar cumulative risk in both populations (83% vs. 86%, respectively; $P=0.89$).

Metabolic factors can stratify the risk of progression to T1D in children with persistent multipositivity

The current DIPP study cohort comprised 218 persistently multipositive children (129 males, 59.2%) who had undergone at least one IVGTT by December 31, 2005 (Publication IV, Table 1). One-hundred and seventeen (53.7%) of these children had

developed T1D by the end of 2008 (progressors) at a median age of 5.3 years (range 2.1-12.5 years). The delay from the initial ICA-based seroconversion to diagnosis varied from 0.5 to 10.7 years. The median follow-up time for children who remained non-diabetic (non-progressors) was 10.2 years (range 4.9-14.2 years). The baseline factors (gender, HLA genotype, INS and PTPN22 gene polymorphisms, and family history of T1D) for progressors and non-progressors were similar, but regarding the pace of the prediabetic disease process, progressors differed from non-progressors by being younger at seroconversion and at the appearance of autoantibody multipositivity, and accordingly being also younger at the IVGTT (Publication IV).

In the fasting state, the differences between progressors and non-progressors were minor, although both plasma glucose and serum insulin concentrations were slightly lower in the progressors (Publication IV, Table 3). After the intravenous glucose infusion, the maximal glucose concentrations were higher and insulin concentrations were lower in the progressors than in the non-progressors. The maximal insulin release is normally reached by the 1-minute sampling after the glucose infusion has been given, and this was also the case in the majority of both progressors (97 out of 117; 82.9%) and non-progressors (90 out of 101; 89.1%). However, the timing of the peak insulin level differed between the progressors and the non-progressors: 17 progressors (14.5%) and only 4 non-progressors (4.0%, $P<0.001$) showed a delayed rise in their insulin levels (peak level reached 5–10 minutes after the glucose infusion), and nine of these 17 progressors (53%) reached their peak insulin level at the 10-minute sampling.

Progressors have lower first-phase insulin release and relative insulin sensitivity than non-progressors soon after the first signs of beta-cell autoimmunity have appeared

The first phase insulin release (FPIR) was lower in the progressors than in the non-progressors (Publication IV, Fig. 3A), and although the younger age of the progressors explained some of the difference in the FPIR values ($r_S=0.44$, $P<0.001$), the correlation between the decreased FPIR and T1D remained significant after adjusting for age ($P<0.001$). FPIR correlated also with the delay from the first IVGTT to diagnosis of T1D ($r_S=0.28$; $P=0.003$) in progressors. FPIR correlated with insulin resistance (HOMA-IR) in both groups (age-adjusted $r_S=0.24$, $P=0.01$ for progressors and $r_S=0.29$, $P=0.004$ for non-progressors), and with relative weight (weight-for height) in progressors ($r_S=0.29$; $P=0.002$), but no correlations were observed between relative height and FPIR.

Insulin resitance was low in both groups, indicating that the persistently multipositive subjects have normal insulin sensitivity in general. HOMA-IR was, however, higher in non-progressors than in progressors (Publication IV, Fig. 3B and Table 3). HOMA-IR correlated with age ($r_S=0.41$) and weight-for-height at the IVGTT ($r_S=0.24$), and maximal glucose ($r_S=0.26$) and insulin concentrations ($r_S=0.36$; $P<0.001$ for all correlations). An inverse correlation was observed between ICA level at the IVGTT and HOMA-IR ($r_S=-0.20$, $P=0.003$). The relative insulin resistance (HOMA-IR/FPIR) that was higher in progressors correlated positively with ICA, IAA, and IA-2A levels at IVGTT ($r_S=0.22$, 0.25, and 0.17; $P<0.01$), and with fasting glucose, fasting insulin, and maximal glucose

level (r_S=0.24, 0.34, and 0.28, respectively), and inversely with the maximal insulin level (r_S= –0.66; *P*<0.001 for all latter correlations).

Changes in relative weight during the prediabetic disease process are minor

Linear growth and weight gain were observed in persistently multipositive DIPP children in the current study. Data on weight and height were available for 197 children (90.4%) for the time preceding the ICA-based seroconversion and for 203 children (93.1%) at and after the seroconversion (Publication IV, Fig. 2A and Table 3). Future progressors had higher relative weight than non-progressors 12 months before the seroconversion, and the correlation between relative weight and T1D remained significant after correction for age at the time of measurement (age-adjusted r_S=0.19, *P*=0.03).

Although minor differences were seen in relative weight prior to seroconversion in both groups, statistically significant differences were observed only in non-progressors and when only subjects who had comparable measurements both before and at seroconversion as well as at the IVGTT (n=187, 85.8%) were included in the analyses. Their relative weight had decreased from the median of 99.2% before seroconversion to 98.5% at seroconversion (*P*=0.03). There were no changes in the relative weight either in the progressors or in the non-progressors when comparing weights at the time of seroconversion and IVGTT (*P*=0.58 for non-progressors and *P*=0.94 for progressors). Eight of the 12 children who were overweight (weight-for-height >120%) at their first IVGTT progressed to T1D, as did all four children who were obese (weight-for-height >140%) at the IVGTT. Three of the obese children had been overweight or obese already 12 months before seroconversion.

Progression to T1D is highly variable even in children with persistent multipositivity

The initial presumption was that persistently multipositive DIPP children would represent a subgroup of children with an estimated 5-year disease risk of approximately 50% and accordingly, would form a uniform study cohort. However, as the delay from the ICA-based seroconversion to diagnosis of T1D varied markedly in the progressors (range 0.5-10.7 years) and the delay from the ICA-based multipositivity to diagnosis of T1D failed to correlate with the age at which this autoantibody status was reached (r_S=0.16, *P*=0.09; Fig. 6), we aimed to assess further factors that could explain this variation in the pace of the prediabetic disease process.

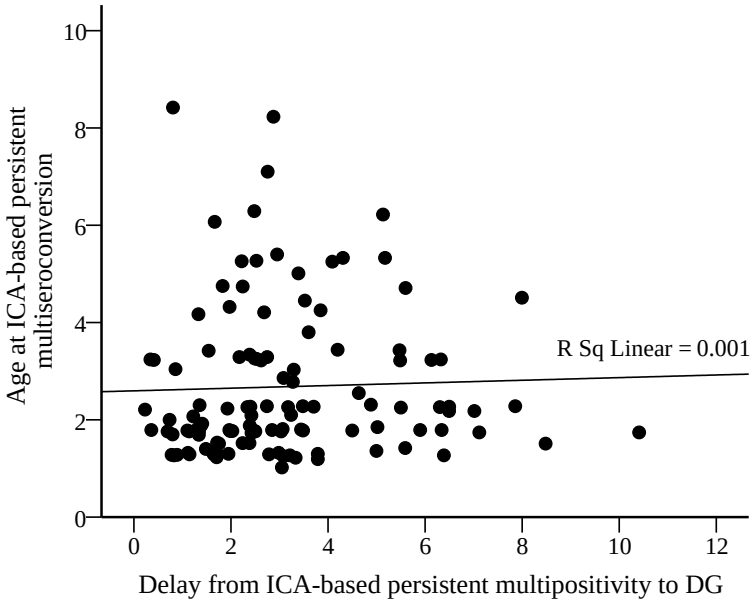

Figure 6. Time interval from ICA-based persistent multipositivity to diagnosis of type 1 diabetes (T1D) in relation to age at which persistent multipositivity was recognized in 218 DIPP children.

The age at diagnosis correlated inversely with relative insulin resistance (r_S= –0.19; *P*=0.04) and maximal IAA level before IVGTT (r_S= –0.46; *P*<0.001), and directly with the age at seroconversion and IVGTT (r_S=0.56 and 0.67, *P*<0.001 for both), as well as with glucose and insulin levels (fasting values r_S=0.20 and 0.24; peak values r_S=0.21 and 0.49, respectively; *P*≤0.02), and with FPIR (r_S=0.46; *P*<0.001), and HOMA-IR (r_S=0.31; *P*=0.01). Those children who did not participate in the intervention trial were slightly older at seroconversion than their peers, but otherwise no other significant differences were observed between the intervention groups or between those with and without intervention treatment (Table 15; partly unpublished data). No correlations were observed between age at diagnosis and any of the genetic factors studied (HLA, INS and PTPN22 variants), growth-related factors (weight-for-height preceding and at seroconversion or at IVGTT), intervention treatment, or levels of autoantibodies except IA-2A (unpublished data). The only correlation between age at diagnosis and autoantibodies was seen for the maximal IA-2A level during the observation period (r_S=0.35; *P*<0.001; unpublished data).

Table15. Persistently multipositive DIPP children (n=283) according to participation in the intervention trial.

	Nasal insulin	**Placebo**	**Did not participate**
		N (%)	
Total	91 (32.2)	97 (34.3)	95 (33.6)
Gender, males	58 (63.7)	56 (57.7)	55 (57.9)
HLA-DQB1*02/*0302, high risk genotype	36 (39.6)	35 (36.1)	38 (40.0)
T1D affected family members at birth	13 (14.3)	10 (10.3)	8 (8.4)
Seroconversion sample IAA-positive	57 (62.6)	65 (67.0)	52 (54.7)
Seroconversion sample multipositive	44 (48.4)	42 (43.3)	43 (45.3)
Progression to T1D	49 (53.8)	56 (57.7)	40 (42.1)
		Years, median (range)	
Age at diagnosis	4.8 (1.0–12.2)	5.5 (2.1–12.5)	4.8 (1.0–11.6)
Age at seroconversion	1.8 (0.3–10.1)	1.6 (0.5–9.6)	3.0 (0.5–10.6)*
Age at maximal DAA status	3.0 (0.5–10.5)	2.7 (1.0–10.3)	4.8 (1.0–12.5)†
Delay from seroconversion to maximal DAA	0.6 (0–7.2)	0.6 (0–7.4)	0.8 (0–10.0)
Delay from seroconversion to diagnosis of T1D	2.7 (0.5–10.9)	3.3 (0.5–9.0)	2.6 (0.1–7.6)

*P=0.004, †P=0.003.

When all the factors that could potentially predict T1D in these persistently multipositive DIPP children were analyzed with the Cox regression analysis, a reduced hazard ratio (HR) was associated with increasing age at IVGTT (HR=0.78, $CI_{95\%}$ 0.68-0.91), and increasing FPIR (HR=0.98, $CI_{95\%}$ 0.97-0.99), while higher relative weight at IVGTT (HR=1.04, $CI_{95\%}$ 1.02-1.06), higher IAA level at IVGTT (HR=1.01, $CI_{95\%}$ 1.01-1.02), and higher IA-2A level at IVGTT (HR=1.01, CI95% 1.00-1.01) indicated increased risk of progression to T1D (Publication IV).

SUMMARY OF FINDINGS

The findings of the current work can be summarized as follows:

1. Sensitivity of GADA, IA-2A and their combination is of same magnitude both in siblings of T1D-affected children and in the general population.

2. GADA and IA-2A are both associated with a higher cumulative disease risk in siblings of T1D-affected children than in the general population, whereas combined GADA and IA-2A positivity indicates a similarly high cumulative disease risk in both groups.

3. The combination of HLA and autoantibody screening detects comparable T1D risk levels in the general pediatric population and autoantibody-positive FDRs of affected children.

4. The natural progression rate to clinical T1D is extremely high in young genetically susceptible children testing persistently positive for multiple autoantibodies, including persistent positivity for IAA.

5. IAA affinity does not facilitate the risk assessment for future T1D in young IAA-positive children with HLA-associated disease susceptibility.

6. IAA seem to represent a mature humoral immune response to insulin already when antibodies appear in young children with HLA-defined predisposition to T1D, and no further maturation is observed during the preclinical disease process.

7. Young age, increased weight-for-height, decreased early insulin response, and increased IAA and IA-2A levels predict T1D in young children with HLA-conferred disease susceptibility and advanced beta-cell autoimmunity.

8. Insulin resistance have minor impact on the progression to T1D after the initiation of the disease process in young normal-weight children with HLA-associated disease predisposition.

DISCUSSION

During the current studies it came evident that many of the predictive factors for T1D that are commonly used in the context of first-degree relatives of patients with newly diagnosed T1D are relevant also in the general population-based cohort of young individuals with HLA-associated disease susceptibility. These studies offered conceptual tools for future screening efforts by stratifying the role of risk factors associated with initiation of the prediabetic disease process, as well as those present during advanced beta-cell autoimmunity.

An approach based on screening newborn infants for HLA risk genotypes and combined with regular clinical and autoantibody follow-up of individuals carrying increased genetic risk for T1D is a feasible and effective way to identify future patients with T1D in the general population. It has been estimated that by this strategy maximally 75% of future patients developing clinical T1D can be identified (Kupila et al. 2001). According to the current studies, autoantibody profiles with highly variable disease risks can be identified by defining categories of autoantibody combinations and by including metabolic factors as variables to the risk evaluation. Individuals with extremely high disease risk can be identified by such a strategy.

Strengths and limitations of the current studies

The strengths and limitations of the current studies are related to the study designs of the research projects they are based on, i.e. the DiMe, LASERI, and DIPP studies, and to the demographic and cultural characteristics of Finland, i.e. the relatively small population size (around 5.3 million at the end of 2008; Statistics Finland, Demographic statistics; www.stat.fi) and the possibility, provided by the Finnish Pediatric Diabetes Register, to track down and obtain autoantibody samples at the time of the diagnosis from the majority of progressors who have dropped-out from prediabetic follow-up (Mäkinen et al. 2008). The advantages of the current study populations include the extensive series of children studied, which facilitates the recognition of even the less prevalent risk factors. From the high T1D incidence in the pediatric population of Finland follows that the disease endpoint is reached in comparatively high numbers of children within a limited time frame, which decreases the risk of skewing the results of analyses by time-dependent confounding factors. It has also been shown that population-based screening of genetic susceptibility for T1D, combined with the possibility to participate in a secondary prevention trial in case signs of initiation of the disease process appear, is well accepted in Finland. Families with children carrying increased genetic disease risk also adhere well to frequent follow-up visits, even if these visits include regular blood sampling.

The main limitations of these large population based studies are related to the heterogeneous screening approaches used in the three different study cohorts, DiMe, LASERI, and DIPP, and these differences guided also the selection of the current analyses that could be performed in and between these cohorts. Especially, the possibilities of

comparing factors associated with seroconversion to autoantibody positivity (timing and the effects of the risk genotypes) between DiMe and LASERI cohorts were limited, because the original study approach was focused on the markers representing advanced autoimmunity (GADA and IA-2A), and comparable background data was not available for both cohorts. Regarding the comparisons between the DiMe and LASERI study cohorts there was also an issue of time scale, as the recruitment of the cohorts was not exactly identical, although within an acceptable time frame.

In the DIPP study cohort the effects of the baseline factors (i.e. gender, family history of T1D, HLA genotype, and INS and PTPN22 gene variants) on seroconversion and progression to T1D could be assessed, but fully only in subsets of the whole DIPP cohort. Detailed data on the seroconversion and the four diabetes-associated autoantibodies ICA, IAA, GADA and IA-2A were available for the DIPP cohort, but the strategy of using ICA as a marker of beta-cell autoimmunity limited the assessment of diabetes-related autoimmunity, as subjects testing positive exclusively for autoantibodies other than ICA were omitted while studying the seroconverted subjects. In addition, the use of ICA for primary screening delayed the identification of seroconversion to autoantibody positivity in subjects who seroconverted first to positivity for other autoantibodies than ICA. The delay between the initial seroconversion and the appearance of ICA-positivity was several years in a few cases. This phenomenon was of minor importance in regard to risk assessment, since considering increased disease risk, the spreading of the beta-cell specific autoimmune response is essential, and among individuals with extended delay between the initial seroconversion and ICA-positivity there were mainly subjects with transient and/or single autoantibody positivity. However, in sporadic individuals, in whom the first immune activation against beta cells had not been sufficient to lead to progressive autoimmunity, the second activation, characterized by the appearance of ICA, represented the initiation of the destructive disease process.

The sensitivity of the current ICA-based screening program to identify future patients with T1D would have increased from 86% to 97%, if the analysis of IAA had been added to the initial screening. This would have been important especially for young seroconverted children, in whom 23-41% of the first seropositive samples, even those preceding ICA positivity, were IAA positive. When compared to ICA screening, the combination of ICA and IAA might considerably reduce the number of progressors who test negative for all autoantibodies during their prediabetic process. However, in the current analyses, the main reason for prediabetic seronegativity was dropping out from the follow-up program and only rarely the absence of seroconversion.

In the present series, there were only three progressors who had presented with apparent T1D, but had been negative for all four autoantibodies studied 4-8 months before their diagnosis. Two of these three progressors were positive for at least ICA and IAA at diagnosis, while unfortunately, no autoantibody sample was available from the time of diagnosis from the third child. The remaining 12 of the 15 preclinically seronegative progressors had discontinued participating in regular follow-up visits, and among these subjects the shortest time interval from the last visit to diagnosis was 1.9 years. This observation suggests that in screening programs based on monitoring diabetes-associated autoantibodies in prepubertal children, 2 years should be the maximal sampling interval.

The role of ZnT8A in the identification of otherwise seronegative prediabetic subjects is under investigation at the moment and remains to be defined.

Predictive characteristics of autoantibodies in the three study cohorts

Comparisons of the disease risk estimates between cohorts originating from the general population and from first-degree relatives of patients with T1D were performed for GADA and IA-2A in the context of the DiMe and LASERI studies, but the series of DIPP children having affected family members at birth was not large enough for reliable comparisons between DIPP children with and without FDRs. However, according to the observations in the DIPP cohort, the predictive values of the ICA-based autoantibody combinations are essentially similar to those reported in studies on FDRs (Bingley et al. 1994, Kulmala et al. 1998, Krisher et al. 2003). The predictive characteristics of GADA and IA-2A were highly similar between initially unaffected siblings in the DiMe study and participants in the LASERI study, who represented the Finnish general population. During the 15-year observation period >80% of the double positive subjects developed T1D in both study cohorts, and the disease sensitivities of single GADA or IA-2A positivity and the combination of these autoantibodies were similar in siblings of affected patients and in the general population.

The cumulative disease risk associated with double positivity was also similar in both DiMe and LASERI cohorts, while a higher cumulative disease risk was observed in siblings for single GADA and IA-2A positivity. According to previous studies, the majority of progressors develop multipositivity months to years before disease presentation, and if choosing the combination of GADA and IA-2A for autoantibody screening, all multipositive German and American schoolchildren could be identified (LaGasse et al. 2002, Schlosser et al. 2002). However, GADA, and especially IA-2A, represent a marker of advanced autoimmunity, and the delay from the initial seroconversion to the appearance of GADA and/or IA-2A may take several months (Kimpimäki et al. 2002). This fact indicates that GADA and IA-2A can only play a minor role in the first step of autoantibody screening in young children, among whom the disease process is often rapid. As one of our targets for the future is to provide effective and safe preventive treatment for prediabetic individuals already at early stages of the disease process, the aim of the autoantibody screening is to reliably identify the future progressors as early as possible after the initiation of the autoimmune process.

Predictive factors for T1D among DIPP children

We used the Cox regression analysis to assess the role of risk factors related to early beta-cell specific autoimmunity that may be potentially present when a child is testing positive for ICA for the first time. Based on the whole DIPP cohort, early IAA positivity, high number of positive autoantibodies at seroconversion, a positive family history for T1D, and high IAA and ICA levels were independent predictors of T1D (Fig. 6). As the Cox regression method is relatively sensitive in relation to differences in the baseline factors included in the analysis, various potential predictive models were assessed, but during the analyses the above mentioned factors remained as independent, but relatively weak predictors of T1D.

The role of early and persistent IAA positivity was, however, highly significant in the current DIPP study cohort. As this observation became apparent during the analyses, the role of IAA affinity as a potentially stratifying factor for the disease risk was assessed in IAA-positive DIPP children. As results supporting the usefulness of the analysis of IAA affinity in risk assessment had been reported earlier for older family members of diabetic patients and non-diabetic schoolchildren (Achenbach et al. 2004b, Schlosser et al. 2005a), our presumption was that a high IAA affinity would differentiate young IAA-positive children progressing rapidly to overt T1D from IAA-positive subjects remaining unaffected or progressing at a slower pace to T1D. At the same time we aimed at finding out whether humoral immune response against insulin, the potential primary autoantigen in the disease process leading to T1D, maturates during the prediabetic disease process, or whether the response is mature soon after its initiation. Our findings showed that IAA affinity was high in the majority of the study subjects, and that high IAA affinity did not differentiate rapid progressors from their slowly or non-progressing peers, and that the immune response against insulin is mature already at the beginning of the disease process in young IAA-positive children with HLA-conferred disease susceptibility.

These findings, and the fact that although the progressors and non-progressors were not matched for their non-HLA genotypes initially, they resembled each other also in these respects, and that during the selection of the non-progressors some of the children originally identified as non-progressors did develop T1D before the analyses were performed, raised the question whether the groups were basically too similar for the detection of any differences. Not surprisingly, the only significant observation in terms of IAA and non-HLA polymorphisms in this study cohort was that the protective PTPN22 genotype (TT) seems to be associated with a slightly lower IAA level when compared to the high-risk associated gene variant. We could, however, confirm the findings of previous studies showing that the correlation between IAA levels and affinity is poor, and that high-affinity antibodies are present also in samples with remarkably low IAA levels (Schlosser et al. 2005a). From the point of view of T1D prediction, we observed that an increasing IAA level, which was strongly related to positivity for multiple autoantibodies, was a marker of increased disease risk in the time frame covered.

Positivity for multiple autoantibodies remained a significant predictor of T1D also among our study subjects, and this observation was in concordance with findings from previous studies assessing the role of diabetes-associated autoantibodies in family members of affected patients and in schoolchildren (Bingley et al. 1994, Hummel et al. 2004a). For example, in the DiMe study the siblings who tested positive for at least three autoantibodies (4.6 %) had a 5-year cumulative disease risk of 57%, while the corresponding risk estimate for triple positive DIPP children (n=263, 3.5%) was 51% in the present series. The frequency of multipositivity is, however, higher in FDRs than in the background population, and to find similar numbers of individuals at high risk for progression to T1D (>50% over 5 years), i.e. triple positive subjects, one third more children should be screened from the background population. The screening effort would be still worth it, since these children represent the majority of individuals at risk for T1D, and without preventive measures covering also individuals at risk in the general population, only a proportion of future cases can be prevented.

Given that the progression rate is extremely high among young persistently multipositive children testing positive for IAA, these children might represent a subgroup of prediabetic children for whom even more intensive immunomodulatory treatment, aimed at delaying or preventing overt disease, might be justified in the future. Regarding preventive interventions, the two major challenges for persistently ICA and IAA positive children may, however, be the young age and remarkably rapid progression to overt disease, e.g. for DIPP children having such an autoantibody profile, the median delay from the maximal autoantibody status to diagnosis was 0.4 years and the median age at diagnosis only 2.6 years.

The levels of all four autoantibodies studied were significantly higher in progressors already at seroconversion and the differences became even more distinct by the time when the children had reached their maximal autoantibody status (Table 10). The effect of the third independent predictor of T1D in the Cox regression analysis, i.e. a positive family history for T1D, on the autoantibody levels was significant in relation to all four autoantibodies, but it was even more prominent regarding ICA and IAA (Table 9). Interestingly, in children with advanced beta-cell autoimmunity, baseline factors (genotypes, FDR status and gender) seemed to play a marginal role in explaining the differences observed in the autoantibody levels. Our findings confirmed, however, the previous observations that higher GADA levels associate with female gender (Sabbah et al. 1999, Lindholm et al. 2004) and that the protective INS polymorphisms are associated with lower IAA levels (Haller et al. 2004, Barratt et al. 2004).

In the whole DIPP cohort the disease risk associated with single ICA positivity of low level (<10 JDFU) did not differ significantly from that observed for autoantibody-negative children (0.3% vs. 0.5%, respectively; P=0.70). Increased risk for T1D that was observed for ICA as a risk marker was associated with ICA≥20 JDFU and with simultaneous positivity for other autoantibodies. The two latter findings have also been observed among family members of diabetic patients (Bonifacio et al. 1990, Bingley et al. 1996), and in fact the clear correlation between multipositivity and ICA level, and the differing HRs associated with these markers (1.9 [CI95%1.5-2.4] vs. 1.004 [CI95%1.002-1.006]), suggest that multipositivity as a risk factor is more important than the ICA titer.

Predictive factors in DIPP children with advanced beta-cell autoimmunity

During the assessment of the disease risk associated with different combinations of positive autoantibodies, it became clear that even among children with signs of advanced beta-cell autoimmunity, i.e. persistent multipositivity, the disease risk and the pace of the prediabetic disease progression is highly variable. Previous studies on family members of diabetic patients and in ICA-positive children have shown that markers related to glucose metabolism could be used in the stratification of the disease risk (Bingley et al. 1996, Gungor et al. 2004, Mrena et al. 2006, Barker et al. 2007), and we assumed that differences in these factors could at least in part explain the variation observed among persistently multipositive DIPP children. These children were also eligible for the randomized, double-blinded, and placebo-controlled intervention trial with intranasally administrated insulin. To study the baseline metabolic status of children before starting

the intervention trial and to assess the prognostic value of the metabolic factors among these children, IVGTTs were performed in all voluntary, persistently multipositive DIPP children as soon as possible after the antibody status had been confirmed.

The current observations confirmed that in prediabetic FDRs of patients with T1D, reduced FPIR was associated with increased disease risk also in persistently multipositive DIPP children (Bingley et al. 1996, Mrena et al. 2006, Xu et al. 2007). In general, among progressors, reduced FPIR identified children with rapid progression to overt T1D, whereas children with higher FPIR values experienced a longer subclinical phase and were older when diagnosed with T1D. The individual variation in FPIR values was substantial, and both progressors with remarkably high FPIR levels and non-progressors with low FPIR values were observed.

When assessing the serum insulin concentrations after the glucose infusion, we noticed that progressors reached the maximal insulin level later than non-progressors, and on the average, they could secrete nearly normal amounts of insulin. This functional abnormality was characteristic of progressors, and in the current study cohort, there was only one non-progressor (male with the moderate risk associated HLA genotype) that reached his peak insulin level after the 1-minute sampling. Although FPIR in this boy was <10 mU/l, he has remained non-diabetic for more than 10 years after the first IVGTT. In this case it seems more likely that the low FPIR observed mainly was due to a functional disturbance rather than extensive beta-cell destruction, but studies in mice have shown that considerable beta-cell recovery can occur after the initiation of the prediabetic process (Zorina et al. 2003).

In the Cox regression analysis, indicators that weakly but independently predicted T1D included young age, increased relative weight, reduced FPIR, and elevated IAA and IA-2A levels. Although in this statistical model insulin resistance and relative insulin resistance failed to predict T1D, both were related to significant predictors of T1D; insulin resistance to weight-for-height and relative insulin resistance to FPIR. These observations support the findings of the ENDIT study and the British study on identical twins in which HOMA-IR predicted T1D in subjects with reduced FPIR (Hawa et al. 2005, Bingley et al. 2008). Similar findings were reported also in the DiMe and DPT-1 studies in which both HOMA-IR and FPIR/HOMA-IR were significantly associated with progression to T1D (Mrena et al. 2006, Xu et al. 2007). These findings indicate that while insulin resistance by itself can cause only mild disturbances in glucose metabolism in young normal weight individuals with malfunctional insulin release, decreased insulin sensitivity may promote progression towards T1D. The role of the obesity-related insulin resistance for the risk of T1D seemed marginal in the current study, and in fact, the average insulin resistance indexes were lower in progressors than in non-progressors. However, among the two progressors and five non-progressors who had elevated HOMA-IR values, progressors were obese and had higher relative insulin resistance indexes than non-progressors. Except for these two cases, no clinically significant long-term changes in the growth-related factors were observed.

When analyzing factors that predispose Finnish children to T1D, we observed that in subjects with HLA-conferred disease risk and signs of advanced beta-cell autoimmunity

the baseline factors, such as gender, genetic risk factors, and family history of T1D played minor roles in the prediction of T1D after the initiation of the diabetic disease process. This phenomenon might be caused by the selection of subjects with similar genetic background, but it may also indicate that these baseline factors contribute mainly to the initiation of the disease process and become less significant as the autoimmunity-mediated beta-cell destruction proceeds.

Prediction and prevention of type 1 diabetes: Practical and ethical issues

Screening programs aimed at identifying individuals at an increased risk for T1D have affected already the life of more than 100 000 Finnish families, and internationally manifolds. During the years of extensive diabetes-related studies the general knowledge of T1D has increased, which has in turn changed the clinical profile of newly diagnosed cases of T1D (Barker et al. 2004, Hekkala et al. 2007). Today the proportion of patients with severe, life-threatening ketoacidosis at diagnosis is smaller, and in the follow-up programs even asymptomatic but diabetic subjects with no metabolic derangements can be identified. A good metabolic balance at the beginning of the disease may preserve some of the endogenous insulin secretion, which in turn is associated with better glycemic control and decreased risk of microvascular complications in the years to come. A metabolically balanced beginning of the illness may also provide a good starting point for learning the basics of insulin therapy, but on the other hand, may sometimes give a slightly skewed conception of the seriousness of this disease, which still remains the most common of the chronic, severe, potentially life-threatening illnesses of childhood and adolescence.

Diabetes, like any other chronic illness, is associated with burdens of various types (Aanstoot et al. 2007). For example, in the USA the productivity loss associated with T1D were estimated to equal about a one third reduction in earnings (Ng et al. 2001), and in Finland the total costs of medication for individuals with diabetes were assessed to be 3.5 times higher than those for the non-diabetic control subjects (Reunanen et al. 2000). For comparison, the estimated costs for a genetically targeted 10-year prediction program in Finland were estimated to be 245 US Dollars, less than 200 Euros per child (Hahl et al. 1998), while the costs for T1D prevention program for 2 years were estimated to be around 1500 Euros per child (Hahl et al. 2003). However, as to date no safe and effective prevention measures have been developed, the ultimate costs of a feasible prediction and prevention program remain merely speculative.

Monetary factors play a substantial role when medical care and population-based prevention strategies are considered, but one should also take into account the psychological effects related to both prevention programs and to the disease itself. According to studies on the psychological impact of genetic and autoantibody screening on the participating families, the knowledge of increased genetic disease susceptibility appears to induce mild anxiety in most parents (Lernmark et al. 2004, Simonen et al. 2006), but on an average, autoantibody testing reduces the anxiety levels of the families, at least in those families who already have affected members (Hummel et al. 2004b). Anxiety and discomfort caused by the prediction programs appear relatively insignificant when compared to the burden caused by T1D: the possibility of developing severe

hypo-/hyperglycemias and long-term complications, efforts needed for reaching optimal metabolic balance, potential employment and career problems, etc. (Hahl et al. 2002, Wasserman and Trifonova 2006).

When considering various aspects of predictive programs aimed at identifying individuals at risk for T1D one has to keep in mind the main goal of all these efforts: finding means to prevent T1D, or at least to delay its clinical presentation. Altogether, both earlier experiences from prospective follow-up studies and the work presented in the current thesis show that it is possible and feasible to genetically screen newborn infants from the general population for HLA risk genotypes, to arrange follow-up for children with HLA-conferred diabetes susceptibility, and to identify individuals who develop progressive beta-cell autoimmunity. As soon as safe and effective preventive measures are available, population-based prevention programs may become relevant in high-incidence countries, such as Finland.

CONCLUSIONS

The rapidly accumulating knowledge of the natural history of T1D has paved the way for identifying the majority of future patients with T1D. By combining genetic, immunological and metabolic data, prediction of T1D will reach the efficacy and reliability required for clinical use. The work presented in this thesis aimed at assessing the immunological and metabolic factors associated with an increased risk for T1D in affected families and in the general population. The results show that, in a population-based cohort of children carrying HLA-conferred disease susceptibility, prospective screening of diabetes-associated autoantibodies results in the identification of individuals at high risk for T1D, and that the estimation of disease risk can be stratified by assessing metabolic markers including circulating glucose and insulin concentration in prediabetic individuals. The risk estimates associated with positivity for multiple autoantibodies for children recruited from the general population, carrying HLA-conferred disease susceptibility, are similar to those observed in family members of patients with T1D. Accurate prediction of T1D is a prerequisite for secondary prevention of this disease. Identifying the right individuals for the right treatment at the right time may in the end save time, money, and resources, as well as alleviate the burden caused by T1D.

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