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Beta-cell Autoimmunity and HLA-Conferred Susceptibility to Type 1 Diabetes among Finnish Children

Relation to Age, Family Constellation and Geographical Area

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ACADEMIC DISSERTATION

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To my husband Janne and our little Emmi

Contents

List of original communications

The study is based on the following original publications referred to in the text by their Roman numerals (I-IV).

I Kukko M, Kimpimäki T, Kulmala P, Kupila A, Korhonen S, Simell T, Ilonen J, Simell O and Knip M (2005): Dynamics of diabetes-associated autoantibodies in young children with human leukocyte antigen-conferred risk of type 1 diabetes recruited from the general population. J Clin Endocrinol Metab 90: 2712-2717.

II Kukko M, Kimpimäki T, Kupila A, Korhonen S, Kulmala P, Savola K, Simell T, Muona P, Ilonen J, Simell O and Knip M (2003): Signs of beta-cell autoimmunity and HLA-defined diabetes susceptibility in the Finnish population: the sib cohort from the Type 1 Diabetes Prediction and Prevention study. Diabetologia 46: 65-70

III Kukko M, Toivonen A, Kupila A, Korhonen S, Keskinen P, Veijola R, Virtanen S, Ilonen J, Simell O, Knip M (2005): Familial clustering of beta-cell autoimmunity in initially non-diabetic children. Diabetes Metab Res Rev 21: in press

IV Kukko M, Virtanen SM, Toivonen A, Erkkilä S, Korhonen S, Ilonen J, Simell O and Knip M (2004): Geographic variation in risk HLA-DQB1 genotypes for type 1 diabetes and signs of beta-cell autoimmunity in a high-incidence country. Diabetes Care 27: 676-681.

Abbreviations

APC	antigen-presenting cells
CDC	Center for Disease Control
CI	confidence interval
CNS	central nervous system
DASP	Diabetes Autoantibody Standardization Program
DAISY	Diabetes Autoimmunity Study in the Young
DENIS	Deutsche Nicotinamide Intervention Study
DiMe	Childhood Diabetes in Finland Study
DIPP	Type 1 Diabetes Prediction and Prevention Project
DPT-1	Diabetes Prevention Trial -Type 1
ENDIT	European Nicotinamide Diabetes Intervention Trial
GAD	the 65 kD isoform of glutamic acid decarboxylase
GADA	antibodies to GAD
GALT	gut-associated lymphoid tissue
HLA	human leukocyte antigen
HR	hazard ratio
IAA	insulin autoantibodies
IA-2	the protein tyrosine phosphatase-related islet antigen 2 molecule
IA-2A	antibodies to the protein tyrosine phosphatase-related IA-2 molecule
ICA	islet cell antibodies
IDDM	insulin-dependent diabetes mellitus
IL	interleukin
IVGTT	intravenous glucose tolerance test
JDFU	Juvenile Diabetes Foundation Units
MHC	major histocompability complex
NOD	non-obese diabetic
OGTT	oral glucose tolerance test
PPV	positive predictive value
RU	relative units
T1D	type 1 diabetes
TRIGR	Trial to Reduce IDDM in the Genetically at Risk

Abstract

Marika Kuuskeri: Beta-cell autoimmunity and HLA-conferred susceptibility to type 1 diabetes among Finnish children: relation to age, family constellation and geographical area

Type 1 diabetes (T1D) is one of the most common chronic diseases of childhood and adolescence. The disease is considered to be of autoimmune origin. Its clinical manifestation represents end-stage insulitis, being a consequence of progressive destruction of the insulin-producing beta cells in the islets of Langerhans after an asymptomatic period of variable duration. T1D is a multifactorial disease with a strong genetic predisposition, the most important contributory genes being located in the HLA-DQ locus on the short arm of chromosome 6, 6p21. Autoantibodies can be detected in normoglycemic preclinical subjects and are used as markers of beta-cell autoimmunity. The present patient series was derived from the population-based Diabetes Prediction and Prevention Project (DIPP), which was initiated in 1994 to find optimal ways to identify children with an increased genetic risk of developing overt T1D from among the general population and to establish effective tools for preventing or delaying progression to the clinical disease.

Genetic screening was performed on infants born at the university hospitals of Turku, Oulu and Tampere, and those with HLA DQB1-defined susceptibility to T1D were invited for an immunological follow-up. If persistent markers of beta-cell autoimmunity appeared, the family was offered the opportunity of enrolling the child for a randomized, double-blinded, controlled intervention trial with daily administration of intranasal insulin.

HLA DQB1 genotypes were observed to be powerful determinants of beta-cell autoimmunity, affecting both the quantity and quality of autoimmunity. Autoantibodies appeared early in life and their frequency increased at least up to the age of 5 years. There was clear geographical variation in the cumulative incidence of autoantibodies among the regions studied here which could not be explained by differences in HLA DQB1genotypes or in the frequency of affected first-degree relatives. The appearance of autoantibodies also showed clear familial aggregation. ICA were observed to be a sensitive marker of beta-cell autoimmunity in our hands, showing good stability and identifying all those with a destructive beta-cell process, i.e. those who progressed to clinical diabetes. The number of progressors was limited, but the preliminary results indicated that IA-2A were the most specific autoantibody reactivity and also had the highest positive predictive value (PPV).

The present experiences provide support for the relevance of grading diabetes risk in relation to HLA DQB1 genotypes, as this risk correlated well with the appearance of autoantibodies. Sequential monitoring of diabetes-associated autoantibodies makes it possible to identify high-risk individuals at a young age, facilitating the initiation of preventive trials and possibly preventive measures in the future, early in the disease process. The geographical difference in the appearance of autoantibodies might be explained by environmental factors. This population should be followed up further to assess whether the same difference can also be seen in the incidence of clinical disease

and to evaluate whether possible environmental factors that either protect the children from the autoimmune process or predispose them to it might vary on a regional basis. A clear familial aggregation of autoantibodies was already seen in the present population of young children, and the families having at least two children with markers of beta-cell autoimmunity should be observed further in order to define the factors that distinguish non-progressors from progressors. ICA analyzed in a laboratory with a high standard of quality control showed high sensitivity in identifying children with destructive beta-cell autoimmunity and were stable enough, and therefore, in our experience, ICA are still the most sensitive of the single autoantibody reactivities for screening for beta-cell autoimmunity. IA-2A are highly specific autoantibodies, but they cannot be used as a screening tool alone due to their rather poor sensitivity.

Tiivistelmä

Marika Kuuskeri: Beeta-soluautoimmuniteetti ja HLA genotyypin välittämä alttius tyypin 1 diabetekselle väestöä edustavilla lapsilla: yhteys ikään ja maantieteelliseen sijaintiin sekä perheittäinen kasautuminen

Tyypin 1 diabetes on yksi yleisimmistä lapsuus- ja nuoruusiän kroonisista sairauksista, ja sitä pidetään autoimmuunitautina. Kliininen tauti edustaa loppuvaiheen insuliittia, joka on seurausta vaihtelevan pituisesta oireettomasti etenevästä beeta-solutuhosta haiman Langerhansin saarekkeissa. Tyypin 1 diabetes on monitekijäinen sairaus, jolla on vahva geneettinen tausta. Tärkeimmät altistavat geenit sijaitsevat HLA DQ-alueella kuudennen kromosomi lyhyessä haarassa. Autovasta-aineita voidaan todeta prekliinisessä vaiheessa lapsilla, jotka ovat vielä normoglykeemisiä ja näitä voidaan käyttää merkkinä beeta-soluautoimmuniteetista.

Tämän tutkimuksen aineisto on peräisin väestöpohjaisesta diabeteksen ennustamiseen ja ehkäisyyn tähtäävästä tutkimuksesta, DIPP projektista. Projekti alkoi 1994 tarkoituksenaan löytää sopivia tapoja seuloa väestöstä ne lapset jotka ovat merkittävässä riskissä sairastua diabetekseen. Tämän lisäksi tavoitteena on löytää keinoja, joilla pystyttäisiin joko kokonaan ehkäisemään kliinisen diabeteksen puhkeaminen tai ainakin lykkäämään sitä.

Geneettinen seulonta suoritetaan niille vastasyntyneille, jotka syntyvät Turun, Oulun ja Tampereen yliopistollisissa sairaaloissa. Ne lapset joilla on HLA DQB1-genotyyppiin liittyvä lisääntynyt riski sairastua tyypin 1 diabetekseen, kutsutaan immunologiseen seurantaan. Jos tässä seurannassa ilmaantuu merkkejä beeta-soluautoimmuniteetista, perheen lapselle tarjotaan mahdollisuutta osallistua satunnaistettuun, kontrolloituun hoitotutkimukseen jossa arvioidaan voidaanko diabetesprosessin etenemiseen vaikuttaa insuliinin päivittäisellä annostelulla nenän kautta.

Väestöä edustavilla lapsilla HLA DQB1 genotyypeillä on merkittävä osa beeta-soluautoimmuniteetin kehittymisessä. Näillä genotyypeillä on vaikutusta sekä autoimmuniteetin määrään että laatuun. Autovasta-aineet ilmaantuivat varhaisessa iässä ja näyttivät lisääntyvän ainakin viiteen ikävuoteen asti. DIPP:n sisällä näkyi selvä maantieteellinen ero vasta-aineiden ilmaantumisessa. Tätä ei voitu selittää eroilla HLA DQB1 genotyypeissä tai diabetesta sairastavien perheenjäsenten osuudessa. Vasta-aineiden ilmaantumisessa nähtiin selvä vasta-aineiden perheittäinen kasaantuminen. Havaitsimme myös, että ICA on herkkä, mutta hyvän stabiliteetin omaava vasta-aine, ja sen avulla löydettiin kaikki ne lapset, joilla oli destruktiivinen beeta-soluautoimmuniteetti, eli jotka etenivät kliiniseen tyypin 1 diabetekseen asti. Kliiniseen diabetekseen edenneiden määrä oli tässä aineistossa pieni, mutta alustavien tulosten mukaan IA-2A oli kaikista tarkin yksittäisistä autovasta-aineista, ja niillä oli myös korkein positiivinen ennustearvo.

Tulokset viittaavat siihen, että käyttämämme geneettinen seulonta on osuva, koska HLA DQB1-riskiluokat korreloivat hyvin autovasta-aineiden ilmaantumiseen. Näkemämme autovasta-aineiden kehityksen mukaan ne lapset joilla on merkittävä riski sairastua tyypin 1 diabetekseen voidaan löytää jo nuoressa iässä, jolloin diabeteksen ehkäisyyn tähtäävät

hoitotutkimukset, ja tulevaisuudessa mahdolliset ehkäisevät toimenpiteet, voidaan aloittaa ajoissa. Maantieteellinen ero autovasta-aineiden ilmaantumisessa voitaneen selittää ympäristötekijöillä. Tätä kohorttia on syytä seurata pidemmälle, jotta voidaan selvittää esiintyykö vastaavaa eroa myös kliinisen diabeteksen ilmaantumisessa. Tällöin voidaan myös selvittää alueiden välisiä eroja mahdollisissa suojaavissa tai altistavissa ympäristötekijöissä. Jo hyvin nuorten lasten joukossa nähtiin selvä autovasta-aineiden perheittäinen kasautuminen. Myös näitä perheitä joissa on vähintään kaksi lasta joilla on merkkejä autoimmuniteetista tulisi edelleen seurata, jotta pystyttäisiin tunnistamaan ne tekijät jotka erottavat toisistaan ne lapset jotka eivät etene ja ne jotka etenevät kliiniseen diabetekseen asti. Saarekesoluvasta-aineet (islet cell antibodies, ICA) löysivät hyvin ne lapset, joilla oli destruktiivinen autoimmuniteetti. Saarekesoluvasta-aineet olivat myös tarpeeksi vakaa vasta-ainereaktiviteetti. Siksi saarekesoluvasta-aineet ovat kokemuksemme mukaan yksittäisistä vasta-ainereaktiviteeteista edelleen paras beeta-soluautoimmuniteetin seulontaväline. IA-2-vasta-aineet olivat hyvin spesifinen vasta-ainereaktiviteetti, mutta ne eivät yksin sovellu beeta-soluautoimmuniteetin seulontaan, koska niiden sensitiivisyys oli melko vaatimaton.

1 Introduction

Type 1 diabetes (T1D) is one of the most common chronic diseases of young children and adolescents (Menon and Sperling 1988). In about two thirds of cases it is diagnosed before the age of 20 years (Anonymous 1988). Its clinical manifestation represents end-stage insulitis, which occurs after asymptomatic destruction of the insulin-producing beta cells in the pancreatic islets of Langerhans. The duration of this subclinical phase, during which T1D-associated autoantibodies can be detected in individuals who are still normoglycemic, can vary from months to years (Thai and Eisenbarth 1993, Knip 1997; see Fig. 1). It has been estimated that around 80-90% of the beta cells have been destroyed by the time of diagnosis, and total beta-cell destruction can usually be seen 1-2 years after the diagnosis in young patients, while in older patients the destruction proceeds more slowly (Foulis et al. 1986).

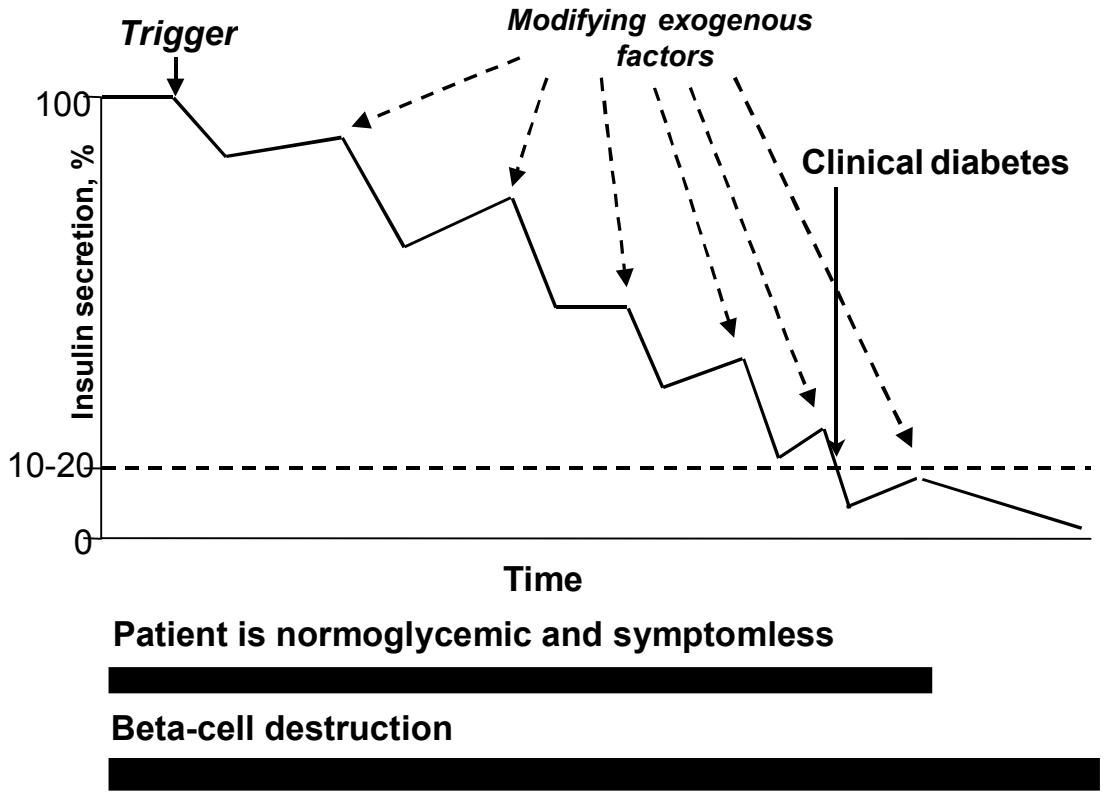

FIGURE 1. Progression from genetic susceptibility to clinical T1D. The disease process may be triggered by an exogenous factor, driven by another environmental determinant and modified by a series of environmental factors in individuals with increased genetic diabetes susceptibility. Associated autoantibodies can be detected before the onset of T1D. Figure modified from Knip (2005).

The disease is considered to be a consequence of an autoimmune process which flares up and declines in intensity repeatedly and gradually destroys the beta cells. This process results in insulin deficiency, followed by clinical symptoms such as polyuria, polydipsia, polyphagia, weight loss and hyperglycemia.

The current consensus is that the disease process is initiated in genetically susceptible individuals when some still unknown triggering factor acts on the beta cells in the pancreas (Eisenbarth 1986, Bottazzo et al. 1990). Among the most extensively studied candidate factors are enterovirus infections (Tauriainen et al. 2003) and early exposure to cow's milk proteins (Knip and Åkerblom 2005). Beta-cell autoimmunity may appear at any age, and in some rare individuals the first signs of autoimmunity may emerge even before birth (Knip 1997). It seems that there is individual variation in the pathomechanisms of diabetes. Unique individual combinations of predisposing and protecting genes and different environmental factors may result in variable outcomes. (Åkerblom et al. 1997) High-risk siblings of children with T1D who develop clinical disease are often characterized by young age, high ICA and IAA levels, reduced insulin secretion and impaired glucose tolerance, but none of these features can completely differentiate siblings who progress to overt disease from those who do not. This also reflects the conspicuous individual variation in the disease process. Linear progression in beta-cell destruction seems to be an exception rather than the rule in preclinical T1D. (Heaton et al. 1987, McCulloch et al. 1990, Thivolet et al. 1991, Knip et al. 1994, Mrena et al. 2003)

T1D is a substantial health care problem in the developed countries, with consequences for both the patients and their families and for society, since it can lead to serious chronic complications, impaired quality of life and reduced life expectancy (Green et al. 1996). Although insulin therapy is designed to mimic endogenous insulin secretion, this cannot be achieved with the current technology, and therefore insulin treatment is always a compromise, the outcome of which is determined by many factors (Menon and Sperling 1988).

The essence of preventing or curing T1D lies in understanding the processes leading to manifest disease and what happens during the long subclinical period. The purpose of this work was to shed more light on these processes. One approach was to describe the role of the HLA DQB1 genotypes in the development of beta-cell autoimmunity and the dynamics of disease-related autoantibodies in children gathered from the general population. The role of HLA DQB1 genotypes in the development of beta-cell autoimmunity has been studied extensively among first-degree relatives of T1D patients in Finland, and it has been shown that these genotypes conferring an increased risk of T1D predispose individuals to the appearance of autoantibodies (Reijonen et al. 1994, Kulmala et al. 2000, Kimpimäki et al. 2000, Kimpimäki et al. 2001). All the subjects considered in the present work were gathered from the general population, in which the dynamics of the autoantibodies concerned have been studied only in children younger than 2 years of age. Kimpimäki et al. (2002) observed a cohort of more than 1,000 subjects with increased HLA-defined disease predisposition derived from the general population up to the age of 2 years, and this cohort was further monitored here for signs of beta-cell autoimmunity up to the age of 5 years. A longer follow-up time of this kind is essential for analyzing the dynamics of beta-cell autoimmunity. Another purpose of the

current work was to expand the perspective by including children carrying HLA genotypes associated with low or decreased susceptibility to T1D.

Although it is well established that clinical T1D shows familial aggregation, there are no data available on whether beta-cell autoimmunity shows familial clustering, and the current work therefore set out to clarify this issue. As environmental factors are considered to be major actors in the initiation and boosting of the beta-cell damage that leads to T1D, it was also of interest to explore whether there are geographical differences in the appearance of signs of beta-cell autoimmunity. A previous Finnish study of first-degree relatives of T1D patients has reported the highest ICA prevalence as occurring in relatives living in the middle part of the country (Eskola et al. 2003). We thus studied the general population here in order to define whether there are geographical differences in the appearance of ICA and multiple autoantibodies, the latter being considered to be a sign of destructive beta-cell autoimmunity. Possible geographical differences in the frequencies of HLA DQB1 genotypes have been addressed earlier by Ilonen et al. (2000) when comparing the frequencies of various genotypes between Turku and Oulu. The analysis was now expanded to include the Tampere region.

In addition to evaluating the predictive characteristics of autoantibodies in relation to clinical T1D in the general population, a further goal of this work was to assess the value of ICA as a means of screening for beta-cell autoimmunity in the general population, since the DIPP project initially used ICA as its primary screening tool. A previous Finnish study on first-degree relatives of T1D patients implied that ICA screening could be replaced with combined screening for IA-2A and GADA (Kulmala et al. 1998).

2 Review of the literature

2.1 Epidemiology of type 1 diabetes

The incidence of T1D in Finland is the highest in the world, but the reason for this is poorly known. Close to 0.7% of Finnish children will present with the overt disease before the age of 15 (Adojaan et al. 1996). Also the incidence has increased in this country, for while Somersalo reported a rate of 12/100,000 children under the age of 15 years in 1953, the figure was 34.6 over the period 1983-1990 and 40.8 over the subsequent 8-year period 1991-1998 (Podar et al. 2001) and has continued to rise in recent years, so that in 2004 it reached the highest incidence ever reported, 54/100,000 (Reunanen A, personal communication).

It has been suggested that an increase in the pool of genetically susceptible individuals may have contributed to the increase in the incidence of T1D, but when Pitkäniemi et al. (2004) set out to test this hypothesis in Finland they concluded that non-Mendelian transmission of diabetic allele(s)/haplotype(s), if present, could explain only a small proportion of the increase in incidence, so that their results emphasized the importance of other, probably environmental factors in modifying the incidence of the disease. The frequencies of risk haplotypes and the high-risk genotype have been observed to be higher and the proportion of patients carrying protective haplotypes or protective genotypes lower among Finnish patients diagnosed before 1965 than among those diagnosed after 1990, indicating that genetic susceptibility has decreased with time and that increasing environmental pressure has resulted in a higher disease progression rate even among subjects with protective HLA genotypes. (Hermann et al. 2003)

The incidence of T1D shows substantial variation worldwide. The DIAMOND study of incidence rates at 100 centres in 50 countries found that the polar-equatorial incidence gradient reported earlier does not appear to be as strong as had previously been assumed, but that the variation seems to reflect the ethnic and racial distribution of the world's population. The overall age-adjusted incidence of T1D was found to vary from 0.1/100,000 per year in China and Venezuela to 36.8/100,000 per year in Sardinia and 36.5/100,000 per year in Finland. This represents a >350-fold variation in incidence among the 100 populations included. Very high incidence rates (≥20/100,000 per year) were also observed in Sweden, Norway, Portugal, the U.K., Canada and New Zealand, while the lowest rates (<1/100,000 per year) were found in China and South America. In most populations, the incidence increased with age and was highest among children aged 10-14 years (Karvonen M et al. 2000).

There is substantial variation in the incidence rate even within Europe. During the period 1989-1994 it varied from 3.2 cases per 100,000 per year in the former Yugoslav Republic of Macedonia to 40.2 cases per 100,000 per year in Finland (Anonymous 2000). Similarly Green and Patterson (2001), reporting on trends in the incidence of T1D in Europe over a 10-year period from 1989 to 1998, found the standardized average annual incidence rate to vary more than tenfold between the centres. Overall, the annual increase in incidence was 3.2%, being highest for children in the age group under 5 years (4.8%) and lowest for children in the 10-14-year age group (2.1%). Central Eastern Europe showed the highest increase, whereas Sardinia and Northern Europe (except Finland)

showed no evidence of an increase. The greatest within-country variation in Europe can be seen in Italy, where the incidence in Lombardy is 6.8/100,000 and that in Sardinia 36/100,000 (Karvonen et al. 1993). In Finland there was a clear geographical variation in the risk for T1D among children under the age of 15 years in 1987-1996, the high-risk areas being found in a wide belt crossing the central part of the country (Rytkönen et al. 2001). Although there is considerable variation in the incidence in Europe, it has been reported that first-degree relatives of patients with T1D from different European countries have similar rates of islet autoimmunity, i.e. susceptibility to the initiation of beta-cell autoimmunity, despite a conspicuous variation in the background incidence of the disease (Williams et al. 2002).

Only about 10% of patients with newly diagnosed T1D have an affected first-degree relative at presentation with the clinical disease (Dahlquist et al. 1989, Tuomilehto et al. 1992). The rest are sporadic cases, whose risk of contracting the disease is 10-15 times lower than that of first-degree relatives (Honeyman et al. 1997). Most studies of the natural history of preclinical diabetes have been performed in first-degree relatives, and it has been questioned how widely such results can be applied to the general population. It seems, however, that the pathological process leading to clinical disease in sporadic cases does not differ from that in familiar cases (Bruining et al. 1989, Veijola et al. 1996).

A series of national registers show that there is a peak in T1D incidence during the pubertal years, between the ages of 10 and 15 years, followed by a rather steep decrease. The explanation could be that those children who are susceptible to T1D encounter the factor/factors provoking the disease during their first 15 years of life, and that either the influence of these factors decreases after that or else a vast proportion of susceptible children have already contracted the disease by the age of 20. (Songini 1998) The pubertal incidence peak seems to be levelling off, however, since the increase in incidence has been highest in the youngest age group, that under 5 years (Green and Patterson 2001).

International comparisons have shown that there is a male preponderance among patients with T1D in countries with a high incidence rate, whereas in low incidence countries there may often be a slight female preponderance (Anonymous 1988, Rewers et al. 1988). This could be explained by sex hormones or by lifestyle factors such as obesity, physical activity or diet, which have been shown to have an influence on the frequency of type 2 diabetes. In addition, one of the possible gene locuses (DX1068) predisposing subjects to T1D has been located on chromosome X, which could indicate that there may be a gender-linked genetic factor contributing to disease susceptibility (Vandewalle et al. 1997). In accordance with the male preponderance in high incidence countries, the incidence rate in Finland has been observed to be 38.4/100 000 in boys and 32.2/100 000 in girls (Tuomilehto et al. 1992).

There is a series of reports indicating that the diabetes incidence rate increases during the cold months in both hemispheres. There were fewer cases diagnosed with T1D during the summer months in Europe during the 10-year period 1989-1998, especially in the 10-14-year age group (Green and Patterson 2001), while a study conducted in Finland pointed to a statistically significant gender difference in seasonality at diagnosis, as there were less new cases among boys in June than at any other time (Karvonen et al. 1996,

Szybinski et al. 1996). Since it is known that T1D has a long subclinical phase before clinical presentation, this seasonal variation in disease onset indicates that there may be some non-specific factor precipitating the clinical disease (Songini 1998).

There are strong indications that the incidence of T1D rises with the socio-economic standard of living and levels of hygiene, although the precise reason why this should be so remains open. A study conducted in Northern Ireland in 1989-1994 reported that the areas with the highest population density, the most material deprivation and the highest incidences of unemployment, rented apartments and household overcrowding had the lowest T1D incidence. Exposure to infections occurs early in life under such conditions, and therefore the programming of the immune system is different from that seen when exposure is delayed. This also hints at a role for infectious agents in the development of T1D (Patterson et al. 1996). A study on the effect of the level of urbanization on T1D incidence in Finnish children showed that the incidence was highest in the rural heartland areas, while the increase in incidence was sharpest in urban areas (Rytkönen et al. 2003). The authors speculated that it is possible that some environmental risk factors have been more prevalent in rural heartland areas than in the rest of the country and that these factors might have increased in urban environments, particularly during the early 1990's.

2.2 Factors predisposing to type 1 diabetes

2.2.1 Genetics of type 1 diabetes

The familial clustering of T1D is considered to reflect the significant contribution of genetic factors to the development of the disease. The presence of clinical T1D in a family member is the strongest known susceptibility factor, although its positive predictive value is not very high. (Foster 1984) An offspring's risk of contracting T1D is around 2% if the mother has the disease and 6% if the father is affected, but increases to 20-30% if both parents have T1D (Foster 1984). A Swedish study reported that a sibling's risk of contracting the disease is about 5%, but if an identical twin is affected, the risk for the other twin is close to 30%. The risk for a non-identical twin is the same as that of other siblings (Fohlman and Friman 1993). The observation that the concordance of T1D is not 100% in identical twins points to the importance of environmental factors in the development of the disease.

T1D is a polygenic disease, and at least 20 chromosomal regions have been linked to T1D susceptibility in humans by genome-wide screening, candidate gene testing and studies of human homologues of mouse susceptibility genes (Pociot and McDermott 2002). Family studies do not show any pattern of Mendelian inheritance, nor is any single genetic polymorphism or mutation consistently implicated (Winter et al. 1993). T1D is not an inherited disease in the classical sense, but rather it is susceptibility to T1D that is inherited (Morwessel 1998).

Although T1D is a polygenic disease, the genetic component has been estimated to explain at most about a half of the lifetime risk of contracting it, according to family and twin studies (Bertrams and Baur 1984, Davies et al. 1994). The remainder of the risk is considered to be mediated by environmental factors.

The most important genes contributing to disease susceptibility are located in the HLA locus on the short arm of chromosome 6, 6p21 (IDDM1) (Bertrams and Baur 1984, Davies et al. 1994). Mathematical models based on twin and family studies indicate that 30-60% of the genetic susceptibility to T1D is due to genes in the HLA region (Rotter and Landaw 1984), the most important being HLA class II alleles (HLA-DR, DQ, DP). HLA molecules are essential for normal antigen recognition, processing and presentation, ultimately leading to the activation and progression of immune responses (Becker 1999). Polymorphisms in class II HLA molecules define which antigen peptides can be bound by them. This process directs thymic maturation and the generation of the T-cell repertoire as well as peptide presentation after antigenic challenge. (Ilonen and Reijonen 1993) The second most important genetic region resides on chromosome 11p15 (IDDM2), which affects the allelic variation in the variable number of tandem repeats (VNTR) at the 5' end of the insulin gene (Bennett et al. 1995, Undlien et al. 1995). IDDM2 is thought to contribute 5-10% of the total genetic susceptibility to T1D (Raffel 1997). Ueda et al. (2003) reported that the gene region encoding the cytotoxic T-lymphocyte antigen 4 (CTLA4) on the long arm of chromosome 2 comprises polymorphisms associated with an increased risk of common autoimmune disorders such as Grave's disease, autoimmune thyroiditis and T1D. An association between T1D and a single nucleotide polymorphism in the PTPN22 gene (C1858T) has recently been described by Bottini et al. (2004). Lymphoid-specific phosphatase encoded by PTPN22 located on the short arm of chromosome 1 is known to be one of the strongest inhibitors of T-cell activation, and this mutation has been shown to reduce the binding affinity of the molecule to its substrate and thereby weaken the inhibitory effect on T-cell activation, possibly conferring susceptibility to T1D. In another recent study, Guo et al. (2004) reported that a single nucleotide polymorphism (A163G) in a newly identified gene encoding the small ubiquitin-like modifier 4 (SUMO4) was associated with T1D. This gene is located in the IDDM5 region on the long arm of chromosome 6. Additional work suggested that the mutant SUMO4 protein could result in stronger stimulated cellular immune responses, which might explain the association with T1D. The potential associations of other chromosomal regions with T1D have so far remained unconfirmed.

The highest risk conferred by HLA is present in subjects who are heterozygous for the DQB1*02 and *0302 alleles, but also the DQB1*0302 allele alone is associated with increased risk, if there are no protective HLA alleles around. The strongest protective allele is DQB1*0602, but also DQB1*0603 confers protection. Subjects with these alleles have a decreased risk of contracting T1D even when they carry the risk allele DQB1*02. The protection provided by DQB1*0301 seems to be passive. It has been shown that the use of only five HLA DQB1 alleles (DQB1*0302, DQB1*02, DQB1*0602, DQB1*0603 and DQB1*0301) is efficient in classifying the risk of T1D and in screening for HLA-conferred susceptibility to T1D in the Finnish population. The HLA DQB1 genotypes can be divided into four risk groups as described in Table 1 (Ilonen et al 1996). The protection or susceptibility provided by HLA is not absolute, for only a fraction of the genetically susceptible subjects progress to clinical T1D (Bruining et al. 1989), and anyone can have signs of beta-cell autoimmunity irrespective of the genetic risk. It can be said, however, that the protection provided by HLA genes prevents most subjects from advancing to progressive beta-cell destruction, while those with HLA susceptibility genes

easily start to proceed towards beta-cell destruction and total beta-cell failure. Aggressive environmental factors and/or a heavy genetic load exerted by genes other than those for HLA can sometimes break the protection conferred by HLA, which explains why some individuals advance to clinical diabetes even though they carry a protective HLA genotype (Knip 1997). The T1D risk associated with HLA genes decreases with age (Noorchashm et al.1997).

There are also indications that the risk associated with a certain genotype may vary between populations. Even within Europe there is heterogeneity in the association of HLA with T1D, DR4-DQA1*0301-DQB1*0302 being the most important risk haplotype in Northern Europe, whereas the DR3-DQA1*0501-DQB1*0201 haplotype is strongly associated with T1D in southern Europe (Ronningen et al. 1993).

	High risk	Moderate risk	Low risk	Decreased risk
HLA DQB1	*02/0302	*0302/x	*0301/0302	*z/z
			*02/0301	*0301/z
			*02/y	*02/0602
			*0302/0602	*02/0603-4
			*0302/0603-4	*0602/z
				*0603-4/z

x= *0302 or a non-defined allele
y= *02 or a non-defined allele
z= non-defined allele

TABLE 1. Classification of HLA DQB1 genotypes according to relative risk of T1D.

2.2.2 Environmental factors

Since it is thought that only about half of the lifetime risk of contracting T1D is caused by genetic factors, environmental factors must play an important role in the pathogenesis of the disease. It is not known specifically which factors contribute to the risk, but there is a series of observations that support a crucial role for such exogenous factors, including: (i) there has been a several-fold increase in the incidence of T1D over the last 50 years in most developed countries; (ii) there has been a simultaneous decrease in the mean age at diagnosis; (iii) only about one third of the identical twins of affected cases present with clinical T1D; (iv) approximately 90% of the patients with newly diagnosed T1D do not have any affected family member (Maclaren and Atkinson 1992); (v) seasonal variation has been observed in the date of diagnosis, with the highest incidence seen during the autumn and winter (Atkinson 1997); and (vi) less than one out of 10 individuals with HLA-conferred susceptibility progress to overt T1D (Knip 1997). There are also reports that the incidence among emigrants from low incidence countries who settle in high incidence countries starts to increase, while the opposite trend has been reported among immigrants from high incidence areas when they move to low incidence areas (Atkinson 1997).

Environmental factors can either trigger the process of beta-cell autoimmunity or accelerate and promote an already ongoing process (Dahlquist 1993). Beta-cell autoimmunity may be initiated at different levels and be due to mechanisms affecting the beta-cell antigen itself, the presentation of the antigen or the regulation of T and B-cell responses (Greenbaum et al. 1994). The progression to clinical T1D can be the consequence of several assaults on the beta cells by environmental factors, each leading to a decrease in insulin secretory capacity and eventually to beta-cell destruction (Knip 1997). In addition, the effects of nutritional risk predictors, for instance, may differ between the fetal period, early infancy and the later years of childhood (Virtanen and Knip 2003). Among the many environmental factors that have been implicated in the development of T1D, infections and nutritional factors are considered to be the most likely determinants.

2.2.3 Viral infections

Viral infections are regarded as major actors in the development of T1D. Enteroviruses are the most frequently quoted group, but there are several other viruses that have been named as factors contributing to progression to clinical diabetes. The role of viral infections in the etiopathogenesis of T1D has been elucidated by serological and epidemiological approaches and by studies of case histories (Szopa et al. 1993).

Viruses may act either through a direct cytolytic effect, by triggering an autoimmune process leading gradually to beta-cell destruction (Yoon 1991), or by molecular mimicry (Kukreja and Maclaren 2000). Enteral infections may also increase the transfer of foreign antigens through the gut mucosa and thereby prime the gut-associated lymphoid tissue (GALT) for sensitization to dietary components. Data from the DIPP study indicate that early enterovirus infections enhance sensitization to bovine insulin in formula-fed infants, which suggests an interaction between two environmental factors (Vaarala et al. 2002).

There are also indications that the timing of the infection is critical for the development of T1D. An infection encountered very early in childhood could protect from T1D, while later infections might on the other hand either initiate or promote the disease process (Patterson et al. 1996). There are also reports of the process towards clinical diabetes starting in utero, so that maternal enterovirus infections during pregnancy may increase the risk of progression to T1D in the offspring (Dahlquist et al. 1995, Hyöty et al. 1995).

The strongest evidence of infections being involved in beta-cell damage and the development of T1D has been gathered with respect to enteroviruses. Based on evidence involving seasonal incidence studies, numerous epidemiological surveys, detailed descriptions of individual clinical cases of an enterovirus infection accompanying presentation with diabetes, and the observation of a temporal relationship between the induction of diabetes-associated autoantibodies and episodes of enterovirus infections, such infections have been implicated as major triggers of the disease process (Hyöty and Taylor 2002). There is also a case report of a baby girl with neonatal insulin-dependent diabetes mellitus who had indications that echovirus 6 induced beta-cell autoimmunity had already started in utero (Otonkoski et al. 2000). Enteroviruses are small, non-enveloped RNA viruses and members of the picornavirus family. The enterovirus subfamily consists of four subgroups: polio viruses, coxsackie B viruses, coxsackie A

viruses and echo viruses, and includes more than 60 distinct serotypes. (Hyöty and Taylor 2002) Some enterovirus serotypes seem to be more capable of causing impaired beta-cell function or death than others. In addition there are observations indicating that the destructive capacity of the virus may not entirely be defined by the serotype, but that some yet unidentified properties of the virus strain may also be involved. (Roivainen et al. 2002) The virus frequently causes viraemia and spreads to many organs, including the pancreas. Most of the infections are mild and subclinical. (Hyöty and Taylor 2002) Although sequential infections with different serotypes, starting from the first few months of life, are common even nowadays (Hyöty and Taylor 2002), it has been suggested that a similar phenomenon as seen with paralytic polio in the early 20th century, is applicable to the role of enteroviruses in T1D. According to this so called polio hypothesis based on the view that a diabetogenic enterovirus infection induces the initial beta-cell damage, the decrease seen in enterovirus infections over the last decades at the population level in highly developed countries may contribute to the increasing incidence of T1D. The protection conferred by transplacentally transferred maternal enterovirus antibodies has become weaker, and in addition the decreased circulation of enteroviruses in the general population leads to a delayed first contact of the offspring with enteroviruses at a time when no protective maternal antibodies are present any longer. This would result in a more severe first infection leading to more frequent complications such as beta-cell damage. (Viskari et al. 2005)

In addition to enterovirus infections, congenital rubella is thought to be associated with diabetes (Hyöty and Taylor 2002). A recent study has indicated, however, that congenital rubella is not associated with any increase in the frequency of beta-cell autoimmunity, raising the possibility that the type of diabetes linked to it is not immune-mediated (Viskari et al. 2003). Mumps has also been associated with T1D for a long time (Harris 1898, Gundersen 1927), but its role is not clear, and the widespread use of mumps vaccines has made it an unlikely contributor to T1D.

The evidence for rotavirus infections is controversial. In a prospective study of children with genetic susceptibility to T1D it was observed that the appearance of diabetes-associated autoantibodies was associated with significant rises in rotavirus antibody titres (Honeyman et al. 2000). A prospective Finnish birth cohort study, however, found no association between rotavirus infections and the initiation of beta-cell autoimmunity in genetically susceptible young children (Blomquist et al. 2002). As there are no other sets of data apart from that mentioned above that reliably link rotavirus infection to T1D, it is unlikely that such infections play an important role in its development.

The role of primary cytomegalovirus infections in promoting or accelerating the development of T1D has also remained open. A Finnish study did not provide any evidence for this (Hiltunen et al.1995), and although an epitope has been identified which is shared by the cytomegalovirus and GAD 65 and seems to be able to induce T-cell cross-reactivity (Hiemstra et al. 2001), the clinical significance of this finding is not clear. The Epstein-Barr virus has been linked to autoimmune diseases, and therefore also to T1D, but although this virus has been associated with occasional cases of diabetes, it is not thought to be a likely candidate for the pathogenesis of T1D (Hyöty and Taylor 2002). Retroviruses have been associated with autoimmune diabetes in animal studies

using NOD mice (Suenaga and Yoon 1988), but reports of retroviruses in humans are not consistent and their role in the development of T1D therefore remains controversial.

2.2.4 Nutritional factors

Several nutritional constituents have been under investigation in order to identify the triggering factor behind T1D. Most attention has been given to breastfeeding and the introduction of cow's milk. Cow's milk differs from human milk in several aspects. First, its protein concentration is higher, principally due to its greater casein content, second, its main whey protein component is beta-lactoglobulin, which is not an endogenous component of human milk, and third, the primary serum albumin amino acid sequence differs from that of human albumin and rodents in a small, circumscribed area (Martin et al. 1991). There are several hypotheses on how cow's milk could be diabetogenic. One theory is that early feeding with cow's milk-based formulas result in immunization to bovine insulin, which differs structurally from human insulin in three amino acid positions (Vaarala et al. 1998, Vaarala et al. 1999). This would suggest that the immune response initially induced by bovine insulin may later be diverted to autoaggressive immunity to beta cells (Knip 2003). This is supported by the observation in prospective birth-cohort studies that insulin autoantibodies (IAA) appear most frequently as the first sign of beta-cell autoimmunity (Ziegler et al. 1999, Kimpimäki et al. 2001). Another hypothesis is that the structural homology between bovine serum albumin and an islet protein p69 could lead to a misdirected immune response to p69 (Karjalainen et al. 1992). It has also been suggested that dietary components may have an effect on gut microbes and GALT. The intestinal barrier function and the immunoregulatory network are poorly developed for a variable period of time after birth (Brandtzaeg 2002), and the introduction of complex cow's milk proteins during this susceptible period could lead to programming of the immune system in a way that favors autoreactivity.

Breastfeeding may offer protection against T1D, for in addition to the above-mentioned protein differences, human milk provides different types and classes of bioactive factors, such as enzymes, hormones and growth factors, anti-inflammatory agents, transporters and digestive enzymes. Some of the components are also involved in the maturation of the infant's gastrointestinal tract. In addition to the passive benefits provided by human milk, several bodies of data support the hypothesis that breastfeeding promotes the development of the infant's own immune system, which may confer long-term benefits. (Rodriguez-Palmero et al. 1999) Shehadeh et al. (2001) hypothesized that human milk provides protection against the development of T1D by virtue of its substantial insulin content. This assumption is supported by the observation that human milk contains high concentrations of insulin, while it is barely detectable in infant formulas. The authors suggested that oral insulin may induce tolerance to insulin and therefore protect the infants against the development of T1D.

Meta-analyses of the literature on the early introduction of cow's milk and short duration of breastfeeding found higher risk ratios for T1D in children who were breast-fed for less than 3 months or exposed to cow's milk before the age of 3 months (Gerstein 1994, Norris and Scott 1996). The role of these two dietary factors as determinants of T1D remains controversial, however.

It has been shown in a German study (Schmid et al. 2004) that delayed exposure to wheat and barley proteins reduces the incidence of diabetes in NOD mice. Presentation with diabetes was delayed and its incidence significantly reduced in female mice that received a wheat and barley protein-free diet throughout life, either from weaning, or from 3 to 10 weeks of age only, but diabetes development was not completely restored by gliadin supplementation. In humans there may be a window of exposure to cereals in infancy outside which initial exposure increases the risk of beta-cell autoimmunity in children with HLA-conferred susceptibility to T1D. In an American birth cohort study (Norris et al. 2003), children initially exposed to cereals either before the age of 3 months or at the age of 7 months or older had an increased risk of beta-cell autoimmunity compared with those who were exposed between the age of 3-6 months, after adjustment for HLA genotype, family history of T1D, ethnicity and maternal age. Meanwhile, a German study of the offspring of mothers or fathers with T1D indicated that early exposure to cereals, i.e. during the first 3 months of life, carries an increased risk of T1D (Ziegler et al. 2003). Such a practice is extremely rare in the Nordic countries, however, where the highest incidence of childhood T1D in the world is to be found.

A Finnish case-control study that explored whether consumption of coffee or tea by the child before the diagnosis of diabetes or by the parents at the time of the child's conception or during pregnancy was associated with a risk of T1D concluded that children who consumed coffee or tea regularly had an increased risk. Parental consumption of coffee or tea at the time of conception and maternal coffee consumption during pregnancy did not affect the risk (Virtanen et al. 1994b). There are also some indications that soy proteins may be linked to T1D, most notably an American study reporting that children who were given soy-based formulas in infancy were more likely to progress to T1D (Fort et al. 1986). There is hardly any evidence linking other food proteins to human T1D.

Dietary N-nitroso compounds (nitrates and nitrites) may be associated with the development of T1D (Helgason and Jonasson 1981, Dahlquist et al. 1990). In a Finnish nation-wide case-control study dietary nitrite intake both by children and mothers was positively associated with the T1D risk independently of the duration of the mother's education, the child's or mother's age, their place of residence or the mother's smoking status. The case mothers received less nitrate from their diet than the control mothers. (Virtanen et al. 1994a) In addition to food, N-nitroso compounds may originate from cigarettes, car interiors and cosmetics (Virtanen and Knip 2003).

2.2.5 Vitamins and micronutrients

A Finnish birth-cohort study found that both regular and irregular vitamin D supplementation in infancy was associated with a decreased frequency of later T1D after adjustment for neonatal, anthropometric and social characteristics (Hyppönen et al. 2001). The DAISY study reported, that maternal intake of vitamin D via food during pregnancy was significantly associated with a decreased risk of beta-cell autoimmunity in the offspring, independently of HLA genotype, family history of T1D, presence of gestational diabetes mellitus, and ethnicity. Vitamin D intake via supplements was not associated with the appearance of beta-cell autoimmunity in the offspring. (Fronczak et al. 2003) The role and use of vitamin D in immunology has been reviewed by Deluca and

Cantorna (2001). Vitamin D receptors are present at significant concentrations in the T lymphocyte and macrophage populations, with their highest concentration in the immature immune cells of the thymus and in mature $CD8^+$ T lymphocytes. Vitamin D compounds either prevent or markedly suppress autoimmune disease in animal models, possibly by stimulating the production of transforming growth factor TGF-beta-1 and interleukin 4, which in turn may suppress inflammatory T-cell activity.

There was no evidence in a Swedish population-based study that vitamin C could influence the risk of developing T1D in childhood (Dahlquist et al. 1990). In contrast, an Australian case-control study showed that the ingestion of vitamin C supplements was less frequent before diagnosis among children who developed T1D than among controls during the corresponding time period (Glatthaar et al. 1988). It is probable that vitamin C has little or no impact on the development of diabetes. The role of vitamin E in the development of T1D has also been considered, but there are no reports that reliably link it with childhood T1D.

In a Swedish case-control study it was observed (Haglund et al. 1996) that a high concentration of zinc in the groundwater was associated with a significant decrease in T1D risk. An even stronger association was seen in small rural areas where the drinking water is taken from local wells and is thus closely related to the local groundwater.

2.2.6 Other environmental risk predictors

Hyppönen et al. (2000) found that both boys and girls in Finland who developed T1D were heavier and taller throughout childhood than their controls. A 10% unit increment in relative weight was associated with a 50-60% increase in the risk of T1D before 3 years of age and a 20-40% increase from 3 to 10 years of age. The increase in T1D risk for a 1 SD score increment in relative height was 20-30%. The authors concluded that the increase in the prevalence of obesity and secular growth that has occurred in most industrialized countries over the last decades may be involved in the increase in T1D incidence. The Eurodiab Study Group reported that accelerated early growth is associated with increased T1D risk in various European populations (Anonymous 2002b). Hyperinsulinemia and increasing insulin demand have been linked to rapid growth, and hyperfunctioning beta cells are more susceptible to cytokine-induced toxicity (Nerup et al. 1988) than resting cells, which could prove to be the link between accelerated growth and T1D.

The association between vaccinations and autoimmune diseases has been under intense discussion. A Danish cohort study found no support for any causal relation between childhood vaccinations and T1D in the general population. The development of T1D in genetically predisposed children (defined as those who had siblings with T1D) was neither significantly associated with the vaccination history. Furthermore, there was no evidence of any clustering of cases 2-4 years after the inoculation with any of the vaccines studied. (Hviid et al. 2004) The German BABYDIAB study similarly failed to detect any association between the development of diabetes-associated autoantibodies or clinical diabetes and the type or quantity of vaccinations, including Bacille-Calmette-Guérin vaccine, haemophilus influenzae vaccine, diphtheria, tetanus and pertussis vaccine, tick-born encephalitis vaccine, and measles, mumps and rubella vaccine

(Hummel et al. 2000), but Vial and Descotes (2004) did point out in a review that a potential link between vaccines and autoimmune diseases cannot definitely be ruled out.

The relationship between atopic disorders and T1D has also been under investigation. A Norwegian population-based case-control study (Stene and Joner 2004) reported that atopic eczema was inversely associated with the risk of T1D after adjustment for age, sex, maternal education, day-care attendance, duration of exclusive breastfeeding and perinatal factors, but that allergic rhino-conjunctivitis and asthma were not significantly associated with T1D. A meta-analysis by Cardwell et al. (2003) indicated that children with T1D have a slight but significant reduction in their risk of asthma, although the findings for other atopic diseases (eczema and allergic rhinitis) were less conclusive. Some early reports may inadvertently have exaggerated the strength of the inverse association between atopic conditions and T1D.

Toxins may promote T1D by modifying or damaging the beta cells, causing the release of autoantigens. Streptomyces is a common soil bacterium that produces many toxic compounds. In an Australian study (Myers et al. 2003), the injection of bafilomycin A1, produced by Streptomyces, into mice impaired their glucose tolerance and reduced the islet size and relative beta-cell mass. It was concluded that exposure to small quantities of bafilomycin in the diet may contribute to the development of T1D. The same group (Hettiarachchi et al. 2004) fed parent NOD mice with sub-toxic doses of bafilomycin in their drinking water from conception until weaning or for various lengths of time after birth and monitored blood glucose in the offspring. Exposure to bafilomycin in utero but not after birth significantly accelerated progression to diabetes and increased the frequency of the disease in the NOD mice.

2.3 Immunology of type 1 diabetes

T1D is regarded as a T-cell mediated autoimmune disease which develops over a highly variable period of time ranging from months to years. During this symptomless preclinical phase the number of beta cells gradually decreases, and it has been estimated that about 80% are damaged by the time of the diagnosis of T1D. In the disease process the islets are infiltrated with inflammatory mononuclear cells (insulitis), including CD8+ cytotoxic T cells. (Kukerja and Maclaren 1999) The cells secreting glucagon, somatostatin and pancreatic polypeptide are generally preserved, but may be redistributed within the islets (Notkins and Lernmark 2001). The best characterized of the immunological processes operative during preclinical diabetes is the development of diabetes-associated autoantibodies, such as islet cell antibodies (ICA), insulin autoantibodies (IAA), antibodies to the 65 kD isoform of glutamic acid decarboxylase (GADA) and antibodies to the protein tyrosine phosphatase related islet antigen 2 molecule (IA-2A).

The most essential gene predisposing an individual to the development of T1D is HLA DQB1. This encodes two HLA-DQ polypeptide chains (α and β-chains), which together form a class HLA II molecule. The most important known role of the HLA molecule is to bind and present either the organism's own peptide antigens or foreign ones to the T-lymphocytes. When foreign peptides are presented, the purpose of the immune system is

to create immunity against infections and other environmental antigens, but when endogenous peptides bind to class II molecules and are presented to the T-cells the purpose is to educate the immune system to distinguish between self and foreign peptides and thus prevent the development of autoimmunity. It is an error in this process that leads to autoimmunity. (Nepom and Kwok 1998)

Many more individuals develop signs of autoimmunity than actually proceed to autoimmune diseases. Less than one out of 10 individuals with the strongest HLA-defined genetic risk of T1D will present with clinical disease (Knip 1997). Kulmala et al. (1998), who studied the predictive characteristics of autoantibodies in siblings of children with T1D, observed that not even all those with four antibody specificities contracted the disease over a 7-year follow-up period, while a few with only one or no antibodies initially did progress to clinical T1D. The risks of clinical disease in siblings with three or four, two, one, or no antibodies was 66, 25, 2 and 0.8%, respectively. Although diabetes-associated autoantibodies do not provide a definitive answer as to who will or will not develop T1D, the nature, intensity and antigenic spreading of the reactivities of diabetes-associated autoantibodies can be of great help in distinguishing individuals who develop diabetes from those who do not (Kukerja and Maclaren 1999).

The scarcity of access to the target organ, i.e. the pancreatic islets, has definitely hampered the work of delineating the pathogenesis of T1D. There are some indications that CD8+ T cells predominate in the islet infiltrates at the time of diagnosis of T1D, but neither the proportion of CD4+ and CD8+ cells nor the significance of other cell types for insulitis (e.g. natural killer cells, macrophages, dendritic cells) has been unequivocally defined (Notkins and Lernmark 2001). The pattern of the immune response in T1D is unclear. There could be a response to a primary autoantigen and then serial responses to secondary antigens, or it may be that there is no single primary autoantigen and autoimmunity to various antigens develops independently and simultaneously (Christie 1996). As a matter of fact, it is not even absolutely clear whether the autoimmunity involved in T1D is the cause or the result of the disease process. It is still possible that an as yet unrecognized combination of genes or environmental factors may trigger the destruction of the beta cells and that the autoimmune response is secondary to that process. (Notkins and Lernmark 2001)

2.3.1 Autoantibodies associated with type 1 diabetes

Screening for diabetes-associated autoantibodies has proved to be an effective way of identifying individuals at risk of contracting the disease. Even though there is a consensus that T1D is a T-cell mediated disease, the appearance of autoantibodies is for the time being the best sign of ongoing beta-cell damage. Although autoantibodies are clearly markers of the disease, it is not known whether they contribute to its pathogenesis or are simply the response to an existing underlying destructive process (Notkins and Lernmark 2001). It has been shown, however, that an individual with no B-cell function is also capable of progressing to clinical T1D, demonstrating that humoral immune responses are not mandatory for the development of the disease (Martin et al. 2001). Antigenic/epitope spreading of the humoral immune-responses is one important marker of impending progression of the disease, because those with only a single autoantibody

progress slowly or mostly remain non-diabetic, whereas those with autoantibodies to multiple antigens most often progress rapidly (Kukreja and Maclaren 1999).

At the time when disease-associated autoantibodies can be detected, the subjects concerned are still normoglycemic. Antibodies can appear already early in life, and prospective data suggest that a greater proportion of children develop autoantibodies between the ages of 9 months and 3 years than at any other time (Atkinson and Eisenbarth 2001). It has been observed earlier in the DIPP study that the first signs of beta-cell autoimmunity in children gathered from the general population and monitored to the age of 2 years may appear already during the first few months of life (Kimpimäki et al. 2002).

There are four autoantibody reactivities which have been confirmed as predicting clinical T1D: ICA, IAA, GADA, and IA-2A (Table 2). ICA, which were first described by Bottazzo et al. in 1974, react with cytoplasmic antigens of all endocrine cells in the pancreatic islets and not only with beta cell components (Dean et al. 1983, Bruining et al. 1984). Both IA-2 and GAD serve as antigens for ICA, but there are still other so far uncharacterized islet cell antigens that also contribute to the appearance of the latter (Noorchasm et al. 1997). ICA can be quantified with a conventional indirect immunofluorescence method on sections of frozen human pancreas (Bottazzo et al. 1974). Although there are indications that isolated ICA positivity is unrelated to the genetic risk of T1D, it has been shown before that ICA have the highest sensitivity for persistent positivity for at least two diabetes-associated autoantibodies (Kimpimäki et al. 2000), which can be regarded as a strong predictive surrogate marker of overt T1D (Kimpimäki et al. 2002). Also, ICA have been observed in family studies to be the most sensitive single autoantibody marker (Bonifacio et al. 1995a, Kulmala et al. 1998). It is for these reasons that they have remained a very useful tool for the identification of subjects at risk of developing T1D, even though there are studies that imply that the ICA assay could be replaced by analyses of antibodies to biochemically characterized autoantigens (i.e. insulin, GAD, and IA-2), particularly the combination of GADA and IA-2A (Bingley et al. 1999, Kulmala et al. 1998, Verge et al. 1996). There seems to be a close relationship between the incidence of T1D and the prevalence of ICA in non-diabetic children in various countries (Adojaan et al. 1996), the highest ICA frequency in non-diabetic children, 4.1%, having been observed in Finland (Karjalainen 1990), the country with the highest incidence rate of the disease. The positive predictive value of ICA is about six times higher in first-degree relatives of patients affected by T1D than in the general population, although a positive predictive value close to this can be achieved in children with no family history of T1D by combining ICA with genetic markers (Bingley et al. 1993). In a Finnish study of siblings of T1D patients (Kulmala et al. 1998) the positive predictive value of ICA was 43.0%, the sensitivity 81.0% and the specificity 95.3%.

Insulin is the only beta-cell specific autoantigen recognized so far. It is a short protein of 51 amino acids encoded by the insulin gene on chromosome 11p15 (Notkins and Lernmark 2001). IAA also recognize proinsulin, and other proinsulin-specific autoantibodies have been observed as well (Kuglin et al. 1988, Keilacker et al. 1995). The highest IAA prevalence (up to 80%) can be seen in young children with newly diagnosed T1D, while only around 20% of adult patients with a recently diagnosed

disease test positive for these autoantibodies (Vardi et al. 1988). IAA are usually the first or among the first autoantibodies to appear in young children (Kimpimäki et al. 2001, Ziegler et al. 1999). Kulmala et al. (1998) reported that the positive predictive value of IAA was 29%, the sensitivity 25% and the specificity 97% among siblings of children with newly diagnosed T1D.

There are two isoforms of GAD (GAD65 and GAD67), of which GAD65 is the main form in human pancreatic islets. GAD is involved in the formation of the neuroinhibitory transmitter gamma aminobutyric acid (GABA). In the islets GAD is mainly situated in the beta cells. It probably plays a role in the inhibition of the secretion of somatostatin and glucagon, and in the regulation of proinsulin synthesis and insulin secretion. (Ellis and Atkinson 1996) GAD is present at high concentrations in the central nervous system (CNS) as well as in the islets, but the blood-brain barrier prevents the invasion of pathogenic antigen-specific T cells into the CNS, thus preventing the development of brain disease (Kukreja and Maclaren 1999). The GAD65 gene is located on chromosome 10p11 and encodes a protein comprising 585 amino acids (Notkins and Lernmark 2001). GADA appear to be more frequent in female patients with T1D and in those diagnosed at the age of 10 years or older (Sabbah et al. 1996). Of the four predictive autoantibodies, it is GADA that have the highest diagnostic sensitivity for T1D in patients over the age of 20 years (Vandewalle et al. 1995). Its positive predictive value in siblings of children with T1D was 42%, its sensitivity 69% and its specificity 96% (Kulmala et al. 1998). It has been proposed that GADA are markers of general autoimmunity rather than of specific beta-cell damage (Knip et al. 2002), a suggestion based on the observations that high GADA levels are associated with the DR3-DQB1*02 haplotype, which is a classical haplotype predisposing carriers to various forms of tissue-specific autoimmunity, that GADA are more frequent in females and that they are often seen in diabetic patients and their first-degree relatives with signs of thyroid autoimmunity.

Antibodies to IA-2 are also known as ICA512. The gene encoding the IA-2 autoantigen is located on chromosome 2q35 (Leslie et al. 1999). IA-2 is a transmembrane protein, which is located in the membranes of the secretory granules in islet cells (both alpha and beta cells) and other neuroendocrine tissues (Solimena et al. 1996, Wasmeier and Hutton 1996, Notkins and Lernmark 2001). There is another protein closely related to IA-2, namely IA-2β (phogrin) (Bonifacio et al. 1995b, Passini et al. 1995, Payton et al. 1995, Hawkes et al. 1996, Lu et al. 1996). Autoantibodies to these two antigens are directed exclusively at their intracellular domains (Leslie et al. 1999). Most sera that recognize IA-2β also recognize IA-2, but not all sera that recognize IA-2 recognize IA-2β, and for this reason IA-2 is the protein used in most immunoassays (Notkins and Lernmark 2001). Like GAD, IA-2 is also present in high concentrations in the brain but the blood brain barrier prevents its invasion into the CNS (Kukreja and Maclaren 1999). The prevalence of IA-2A is highest in those who present with clinical disease before the age of 15 years (Bonifacio et al. 1995b, Christie et al. 1997). It has been suggested that IA-2A may be related to rapid progression to T1D (Gardner et al. 1999, Christie et al. 1994), and there are indications that positivity for these autoantibodies is a more direct predictor of progression to overt diabetes than positivity for multiple autoantibodies *per se* in siblings of patients with T1D (Decochez et al. 2002). Kulmala et al. (1998) obtained a positive predictive value of 55% for IA-2A, a sensitivity of 69% and a specificity of 98%.

	ICA	IAA	GADA	IA-2A
Autoantigen	IA-2, GAD and uncharacterized islet cell antigens	Insulin and proinsulin	the 65 kD isoform of GAD	IA-2
Location of the autoantigen	Pancreatic islets and CNS	Beta cells	Pancreatic islets and CNS	Pancreatic islets and CNS

TABLE 2. Major autoantibodies in T1D and their autoantigens.

2.3.2 Cell mediated autoimmunity

Even though the immunological mechanisms behind T1D are still poorly defined, studies conducted in both non-obese diabetic (NOD) mice and humans indicate that T cells are the main actors in the disease process (Castano and Eisenbarth 1990). Both CD4+ MHC class II T cells and CD8+ MHC class I T cells are involved in the development of diabetes in NOD mice (Wicker et al. 1995), and studies using this animal model have indicated that several cytokines (gamma interferon, interleukins 2,4,5,6 and10 and tumor necrosis factor) play important roles in the disease process leading to T1D, while antigen-presenting cells (APC) and many adhesion molecules are also important (Tisch and McDewitt 1996). The role of cell-mediated immunity is less clear in human type 1 diabetes, but in parallel to NOD mice, it evidently involves beta-cell antigen recognition, possible defects in antigen presentation and/or other cell functions and abnormal levels of expression of mediating molecules. (Atkinson and Maclaren 1994)

Nepom and Kwok (1998) proposed four stages in the development of autoimmune diabetes: 1) the presence of disease-associated HLA molecules leads to the selection of potentially autoreactive T-cell specificities during T-cell development, specificities characteristic of individuals who are genetically at risk, 2) the individual becomes autoimmune-prone by virtue of peripheral amplification of the potentially autoreactive T cells, probably influenced by environmental, infectious and immunological challenges, 3) target organ-specific events and local immune activation occur, creating a setting of actual autoimmunity, including specific antigen recognition by the autoreactive T cells, and 4) it is determined whether this autoimmunity will be self-limited or whether it will progress to clinical disease, since potent regulatory mechanisms are capable of modulating autoimmunity for long periods of time. The failure of these regulatory mechanisms in step four is likely to involve the function of non-MHC genes. Thus the activation of autoreactive T cells does not necessarily lead to the disease.

There are defects in T-cell immunoregulatory functions in autoimmune diabetes both in humans and in NOD mice. The presentation of natural killer (NK) T cells and resting CD4+CD25+ T cells, two important immunoregulatory T cell subsets, has been reported to be reduced both before and after clinical diagnosis, and the peripheral T cells are defective in secreting Th1 cytokines such as INF-γ, suggesting that there are certain broad, intrinsic T-cell defects underlying the disease (Kukreja et al. 2002).

CD4+ T cells can be divided into two subgroups according to their chemokine receptors and the cytokines they secrete, i.e. Th1 and Th2 cells (Kukreja and Maclaren 1999). The function of these subgroups is antagonistic, because they have down-regulatory effects on each other (Mossman and Coffman 1989). Th1 cells mainly support cell-mediated immunity both in humans and in mice and are widely considered to be the CD4+ cells involved in the development of autoimmune diabetes, at least in mice. They also mainly secrete gamma-interferon and interleukin 2. (Tisch and McDewitt 1996, Rabinovich 1994) On the other hand, Th2 cells support humoral responses and secrete interleukin 4, 5 and/or interleukin 13 (Rabinovich 1994). It is believed that beta-cell destruction may be a consequence of the Th1 polarization of antigen-specific immune responses, while Th2 responses are thought to have a regulatory function, which may lead to protection from the development of destructive insulitis (Undlien and Thorsby 2001).

Although the initiation of beta-cell destruction is an open question, the presentation of beta-cell specific autoantigens by APC (macrophages, dendritic cells and B lymphocytes) to CD4+ helper T cells in association with MHC class II molecules is considered to be the first step in this process. These APC secrete IL-12, which promote the differentation of Th0 cells into cells of the Th1 type. The Th1 cells then secrete IL-2 and IFN-γ, which further stimulate macrophages or specific cytotoxic CD8+ T cells to release free radicals and cytokines such as IL-1β and tumor necrosis factor-α, leading to beta-cell apoptosis. Natural killer T cells could prevent beta-cell destruction by secreting IL-4 early in the differentation of Th0 cells, favoring a benign response of the Th2 type and down-regulating the Th1 cells. (Kukreja and Maclaren 1999)

The Th1 and Th2 cytokine shift described above is still questionable and is most likely a simplification of the immunological disease process involved in T1D, although many reports support the model. If it were a question of simple Th1 polarization, strong antigen-specific T-cell responses and low autoantibody levels would be expected in those who present with clinical T1D, whereas high autoantibody levels could be assumed to be protective. An inverse relation has been reported between GAD-specific T-cell responses and GADA in high-risk subjects who subsequently progressed to clinical T1D (Harrison et al. 1993). In addition Serreze et al. (2001) reported that while some immunomodulatory protocols do indeed block the development of autoimmune diabetes in NOD mice through enhancement of Th2 cytokine production by beta-cell autoreactive T cells, such a cytokine shift is in some cases the consequence rather than the cause of protection, and that protection is actually mediated through different mechanisms. Also, cytotoxic T-cell clones of both Th1 and Th2 lineages can be used to transmit diabetes in NOD mice (Kukreja and Maclaren 1999).

2.4 Prediction and prevention of type 1 diabetes

The existence of autoantibody-positive, non-progressing relatives of patients with T1D demonstrates that clinical disease is not inevitable even after immune recognition events have occurred. Disease progression can be halted even after the initiation of the autoimmune stage. (Nepom and Kwok 1998) In order to actively prevent T1D, we would have to be able to define the factors that initiate or promote beta-cell destruction in genetically susceptible individuals and then remove these factors from their environment

and/or reduce the general diabetes risk by altering the environment (Songini 1998). In addition to the fact that we have so far not been able to identify any factors triggering T1D, it is always difficult to alter the environment effectively.

A combination of genetic, immunological and metabolic markers can be used to predict the disease risk in relatives of patients affected by T1D and also in the general population (Morales et al. 2001). Although the long preclinical period enables the recognition of individuals at risk who comprise the target group for preventive measures, there is conspicuous individual variation in the course of preclinical T1D. Autoantibody levels and their numbers can change from time to time even in a given individual, and there can be substantial fluctuation in the disease process even in high-risk subjects. This can complicate the identification of those individuals who will progress to clinical T1D at a time when they still have enough beta-cell function to remain normoglycemic. (Knip et al. 1994)

The risk of developing T1D can be estimated to some extent by performing HLA DR/DQ typing in the general population and unaffected relatives (Tuomilehto 1999). The presence of multiple autoantibodies represents a significant risk of progression to clinical T1D irrespective of which autoantibody combination is present (Maclaren and Lan 1999), and the use of intravenous glucose tolerance testing (IVGTT) can help in assessing a subject's insulin secretory capacity (Morales et al. 2001). By using a combination of these risk markers, individuals can be identified from the general population who have a disease risk as high as that observed among first-degree relatives.

The prevention of T1D can be implemented at three levels (Knip 1992, Pozzilli 1996). Primary prevention comprises strategies that aim at lowering the incidence of the disease by reducing the risk of contracting it. This is based on the possibility of identifying environmental risk factors that can be modified before any signs of beta-cell autoimmunity can be detected. Unfortunately, the options for carrying out primary prevention trials are limited by the fact that there are only a few environmental risk factors with a confirmed impact that can be safely modified in young children. The goal of secondary prevention is to reduce the incidence of T1D by halting ongoing beta-cell damage at a point where there is still a sufficient functional beta-cell reserve. Here the preventive measures are aimed at normoglycemic individuals who already have signs of beta-cell autoimmunity. Tertiary prevention can be implemented after the diagnosis of T1D, the objective being to restore or preserve beta-cell function and to prevent complications of the disease. (Knip and Åkerblom 1998)

Strategies for preventing T1D can be divided into three categories: (i) beta-cell protection (cytokine antagonists, modulation of cytokine signaling, inhibition of the Fas ligand and perforin involved in cell stress-induced apoptosis, anti-apoptotic factors and anti-oxidative stress), (ii) beta-cell regeneration (using beta-cell growth factors and stem-cell technologies on the stem cells in the pancreatic ductal epithelium or the islets of Langerhans (Guz et al. 2001, Garcia-Ocana et al. 2001), and (iii) beta-cell replacement (human whole pancreas or islet transplantation, genetically engineered insulin-secreting cells, automated insulin delivery devices and bioartificial pancreas implants) (Kawasaki et al. 2004).

2.4.1 Animal models of the prevention of type 1 diabetes

Most of the current clinical trials aimed at preventing T1D in humans are based on strategies developed in animal models (Hänninen et al. 2003). The NOD mouse is the most widely used model, and surprisingly many procedures have been reported to delay or decrease the incidence of diabetes in these mice. Another widely used animal model is the bio-breeding (BB) rat. The protocols employed with NOD mice have been based on (i) blocking the activation or function of T-lymphocytes or their subtypes, or their migration into the pancreatic islets, (ii) administration of autoantigens in forms and schedules that are anticipated to enhance the regulation of specific immune responses, and (iii) allocation of numerous antigen-non-specific substances with immunomodulatory effects, or a possible protective effect on beta cells (Hänninen et al. 2003).

Gene therapy is a promising new approach in the field of T1D prevention. Tian et al. (2004) showed that expression of diabetes-resistant MHC class II beta chains in NOD mice following retroviral transduction of autologous genetically engineered bone marrow hematopoietic stem cells prevented the development of autoreactive T cells by intrathymic deletion and protected the mice from the development of insulitis and diabetes. They suggested that this approach could also be beneficial in the context of islet transplantation and could be used to prevent the recurrence of autoimmunity after the transplantation.

Genetic vaccines provide promising prospects for the prevention of T1D. Vaccines may work in various ways by altering the immune response from a destructive one (e.g. Th1) to a more benign one (e.g. Th2), by inducing antigen-specific regulatory T cells, by deleting autoreactive T cells, or by preventing immune cell interaction (Petrovsky et al. 2003). Genetic vaccinations use plasmids that encode an autoantigen (von Herrath and Whitton 2000). DNA-plasmids encoding e.g. insulin, heat shock protein 60 and GAD have been successfully used in NOD mice (Urbanek-Ruis et al. 2001, Quintana et al. 2002, Li and Escher 2003).

Animal models also provide us with information on the possible adverse effects of promising treatments. A study using transgenic mice has indicated that oral exposure to a protein expressed in beta cells may induce autoimmune diabetes. This raises the possibility that the oral administration of beta-cell antigens could potentially accelerate target cell destruction (Blanas et al. 1996), i.e. the administration of oral insulin could lead to beta-cell damage, for instance.

2.4.2 Clinical trials to prevent progression to type 1 diabetes

The intervention modality used in the DIPP study is intranasally applied human insulin, the aim being to test whether mucosal administration of insulin can prevent or delay presentation with T1D. Insulin is currently the only known beta-cell specific autoantigen, and it is also the most inexpensive antigen characterized (Knip 1997). The arguments for choosing insulin also include its safety, availability and practical considerations (Kupila et al. 2001). In a safety study involving healthy adults, intranasal insulin was observed to be well tolerated, the risk of hypoglycemia being minimal. No objective adverse nasal

effects were detected and it did not induce the production of any of the four autoantibodies that are predictive of diabetes (Kupila et al. 2003). On the other hand, there have been suggestions that the mucosal administration of insulin may induce insulin antibodies that could potentially complicate later insulin therapy (Knip 1997). Intranasal antigen administration has also been shown to induce potent cytotoxic T-cell immunity (Gallichan and Rosenthal 1996). In the DIPP study at-risk children (HLA DQB1*02/0302 or *0302/x; x=*0302 or a non-defined allele) derived from among the general population who develop persistent positivity for at least two autoantibodies during a prospective follow-up are invited to be randomized for the intervention trial with intranasal insulin. The intervention is a randomized, placebo-controlled and double-blinded trial. No data on the effects of this treatment are yet available, but intranasally applied insulin is thought to act through the induction of regulatory T cells or the inactivation of pathogenic T cells (Hänninen et al. 2003).

The Diabetes Prevention Trial – Type 1 Diabetes (DPT-1) was another major randomized, controlled clinical trial undertaken to assess whether insulin could prevent or delay T1D in relatives of patients with diabetes (Anonymous 2002a). The study had two parts. The first, a parenteral insulin trial, involved relatives with a projected 5-year risk of diabetes higher than 50%. Subcutaneous insulin was administered to prevent T1D based on the idea of inducing beta-cell rest, resulting in a lower requirement for endogenous insulin secretion and reduced release of islet autoantigens (Hänninen et al. 2003). The results showed that the insulin regimen used did not delay or prevent the development of diabetes. As an explanation for this negative outcome, the investigators claimed that the intervention was initiated too late in the disease process or that the dose was not the optimal one. A long-term follow-up is still going on, however. The second part studied the effect of oral insulin therapy in relatives with a projected 5-year risk of 26-50% for progression to T1D. The overall outcome, as reported recently (Skyler et al. 2005), was negative, with no difference in the rate of progression to clinical T1D between the two groups. In a subanalysis including those relatives with a high initial IAA titer there was a difference of borderline significance, with a reduced rate of progression in the group receiving active treatment. A new intervention trial targeting relatives with high IAA levels is in the planning phase to confirm the above DPT-1 observations.

The Deutsche Nicotinamide Intervention Study (DENIS) was started on the basis of the positive outcome of animal experiments in which nicotinamide had been shown to protect beta cells from inflammatory insulitis. Nicotinamide has many biological effects which can potentially provide beta-cell protection. It inhibits the poly(ADP)ribosepolymerase (PARP) enzyme and thus saturates intracellular nicotinamide adenine dinucleotide (NAD), which prevents beta-cell destruction. In addition, there have been reports that nicotinamide inhibits the production of nitric oxide provoked by cytokines in the islet cells and the expression of MHC class II genes provoked by cytokines in cultured islet cells. (Knip and Åkerblom 1998) The subjects in DENIS were siblings (age 3-12 years) of patients with T1D who had a high ICA titer [$\geq$ 20 Juvenile Diabetes Foundation Units (JDFU)] and hence were considered to have a high risk of developing diabetes within 3 years. The trial failed to show any delay or decrease in the progression to overt disease and was terminated after the second interim analysis. The investigators did not, however, exclude the possibility of a less strong but still potentially meaningful, reduction in the risk for the cohort studied, and stated that a possible major effect of high-dose

nicotinamide might well be limited to individuals with a lower risk of progression to diabetes than the individuals involved in the DENIS study. (Lampeter et al. 1998)

The European Nicotinamide Diabetes Intervention Trial (ENDIT) set out to assess whether high-dose nicotinamide could prevent or delay clinical presentation with diabetes in subjects with a first-degree family history of T1D. This again was a randomized, double-blind placebo-controlled trial, and included relatives with confirmed islet cell antibody (ICA) levels of 20 JDFU or more and a non-diabetic oral glucose tolerance test. Participants were recruited from 18 European countries, Canada, and the USA, and were randomly allocated to receive oral slow-release nicotinamide or a placebo for 5 years. The finding was that nicotinamide was ineffective at the dose used and that there was no difference in the development of diabetes between the treatment groups. (Gale et al. 2004)

PREVEFIN is an Italian pilot study aimed at testing whether cow's milk administered early in life is diabetogenic. Newborn infants from the general population with high-risk HLA-DR/DQ genotypes for T1D (DRB1*03-DQB1*0201/ DRB1*04-DQB1*0302) are being recruited to two treatment protocols from the time when their mothers stop breastfeeding them, or if they do not breast-feed at all. The intervention consists either of normal cow's milk formula combined with vitamin D supplementation or cow's milk hydrolysate with vitamin D supplementation, continued up to the age of 1 year. The appearance of diabetes-associated autoantibodies will be used as an endpoint. Subjects participating in a project called DIABFIN form a control and observational group. These DIABFIN children are also derived from the general population and have the same high-risk HLA genotype as the children in PREVEFIN. (Pozzilli et al. 2003)

The Trial to Prevent Diabetes in the Genetically at Risk (TRIGR) is an international trial that has been designed to determine whether weaning to a highly hydrolyzed formula can reduce the incidence of diabetes by 10 years of age. Approximately 5 000 newborn infants with at least one family member (mother, farther or older sibling) affected by T1D will be HLA genotyped, and those with high-risk alleles (DQB1*0302 and DQB1*0201) will be included in an intervention trial that requires the recruitment of 2032 eligible infants. Breast-feeding is encouraged, but when the mother is ready to start weaning, the subjects will receive either standard cow's milk formula or one containing a non-antigenic, protein hydrolysate until 6 months of age. (Åkerblom et al. 2005) The study is taking place in 12 European countries, 6 centers in the USA, 12 centers in Canada and 3 centers in Australia. The major outcome of the first phase will be the cumulative incidence of diabetes-associated autoantibodies and/or clinical diabetes by the age of 6 years, and that of the second phase will be the cumulative incidence of overt T1D by the age of 10 years. (Åkerblom et al. 2005)

More than 750 T1D patients worldwide have received islet transplantation since 1974. The procedure still faces many obstacles, e.g. the scarcity of donor cells, the adverse effects of immunosuppression and complications in the transplantation procedure, e.g. intra-abdominal complications and transmission of infectious agents. (Rother and Harlan 2004) Thus islet cell transplantation cannot be said to be the answer to the question of how to prevent T1D for the time being, but if further research succeeds in providing

unlimited access to insulin-producing cells for transplantation without the need for immunosuppression, the scenario will change dramatically.

3 Aims of the research

The purposes of this work were:

1. to assess the role of HLA-defined genetic disease susceptibility and protection in the appearance of signs of beta-cell autoimmunity in a series of children derived from the general population;
2. to study the natural history of beta-cell autoimmunity in children with increased genetic T1D susceptibility derived from the general population;
3. to study the possible familial and geographical clustering of beta-cell autoimmunity in children with HLA-conferred susceptibility to T1D derived from the general population;
4. to examine the feasibility of ICA as a screening tool for autoimmunity in the DIPP setting in genetically susceptible children derived from the general population; and
5. to study the relationship between humoral signs of beta-cell autoimmunity and clinical T1D.

4 Subjects and methods

4.1 Subjects

The series described in all publications were derived from the Finnish Type I Diabetes Prediction and Prevention Project (DIPP), a population-based study aimed at identifying newborn infants who carry HLA-conferred susceptibility to T1D (phase I), recognising at an early stage the appearance of markers of autoimmunity known to precede clinical disease in those with genetic susceptibility (phase II), and delaying the onset of clinical disease in those at genetic risk with signs of beta-cell specific autoimmunity (phase III) (Kupila et al. 2001).

HLA screening of newborn infants was initiated at Turku University Hospital in November 1994, at Oulu University Hospital in September 1995 and at Tampere University Hospital in October 1997. Cord blood samples were obtained from all the infants born in these three university hospitals, and the families received oral and written information on T1D and the DIPP study. Families where neither of the parents was of Caucasian origin (more than 99% of the Finnish population are Caucasian), where the parents had difficulties in understanding Finnish, Swedish or English or where the newborn infant had a severe congenital disease were excluded. Of the eligible families, 94% gave their informed consent to genetic screening. Families with an infant carrying increased HLA-conferred susceptibility to T1D were invited for observation and immunological surveillance for the emergence of diabetes-associated autoantibodies and development of T1D. Among the infants tested, 14.8% carried the increased risk genotypes and approximately 80% of their parents gave their consent for participation in the follow-up study. (Kupila et al. 2001) The unaffected older siblings of the genetically susceptible infants (index cases) were also invited for genetic screening. If the sibs had susceptibility genotypes they were invited to take part in the immunological follow-up.

ICA (islet cell antibodies) were used as the primary screening tool for beta-cell autoimmunity. Where individuals became positive for ICA, all their preceding and subsequent samples were also analyzed for antibodies to the 65 kD isoform of glutamic acid decarboxylase (GADA), antibodies to the protein tyrosine phosphatase-related islet antigen 2 molecule (IA-2A), and insulin autoantibodies (IAA). Serum samples for the immunological surveillance were taken at ages of 3, 6, 12, 18 and 24 months and at intervals of 6-12 months thereafter. If the child became positive for one or more autoantibodies the time interval between the serum samples was reduced to 3 months. Maternal antibodies, defined by being present in cord blood, decreasing thereafter and disappearing from the child's peripheral circulation by the age of 15 months at the latest (Kimpimäki et al. 2002), were excluded from the analyses.

Children who tested positive for multiple (≥2) autoantibodies in two consecutive samples were invited to take part in a randomized, double-blinded, placebo-controlled prevention trial with nasal insulin to assess whether mucosal administration of insulin can prevent or delay the clinical manifestation of T1D. Both an IVGTT (intravenous glucose tolerance test) and an OGTT (oral glucose tolerance test) were performed before the start of the intervention and annually thereafter.

The DIPP protocol was approved by the ethical committees of the three participating hospitals. Informed consent was obtained from the parents or guardians of the children in three steps: first for genetic screening from cord blood, second for the follow-up of children with HLA-conferred disease susceptibility, and third for participation in the intervention trial. The investigation was performed according to the principles of the Declaration of Helsinki.

4.1.1 Subjects in the substudies

The series discussed in paper I included the first 1,006 index cases of the DIPP study (533 boys, 53.0%), born between November 1994 and July 1997 and observed at least to the age of 2 years, for whom all the samples available by the age of 5 years had been analyzed for ICA, IAA, GADA and IA-2A. Of these children 797 (79.2%) remained in the follow-up up to the age of 5 years, 252 (25.0%) carried the high-risk genotype and 754 (75.0%) any of the moderate-risk genotypes. The same population had been studied previously by Kimpimäki et al. (2002) up to the age of two years.

The population in paper II consisted of 1,584 older sibs (783 males, 49.4%) who had been enrolled by the beginning of June 1999. Their mean age was 6.2 years (SD 3.5, range 1.2-24.1 years). The series also included sibs with low or decreased HLA-conferred disease risk.

The series for paper III comprised 2,283 families with at least two children (the index case and a minimum of one older sib) who had given a blood sample for genotyping and at least one blood sample for the analysis of diabetes-associated autoantibodies. There were altogether 5,836 children in these families (3,074 males, 52.7%), the median number per family being two (range 2-13). The age at sampling varied from 0 (cord blood sample) to 24.1 years (median age 2.2 years). If a sample was found to be positive for ICA, all preceding and subsequent samples from that individual were also analyzed for GADA, IA-2A and IAA. In addition, all siblings of the ICA-positive index cases were tested for all four autoantibodies. The analysis in this paper also includes sibs with low or decreased HLA-conferred susceptibility.

The population in paper IV comprised 4,642 index cases from the three DIPP regions of Finland (the Turku, Oulu and Tampere regions), and comparisons of the genetic risk and development of autoantibodies were made between the children born in these three regions. The subjects were born between November 1994 and November 2000, and 2,447 of them (52.7%) were boys. The analysis was based on samples taken before the end of March 2001.

Paper	Subjects	N
I	Genetically susceptible index cases	1,006
II	Older sibs in all genetic risk groups	1,584
III	Children in families with at least two children who had been genotyped and who had given at least one blood sample for the analysis of antibodies, children from all genetic risk groups	5,836
IV	Genetically susceptible index cases	4,642

TABLE 3. Populations discussed in the substudies.

4.2 Methods

4.2.1 Genetic screening

HLA-DQB1 typing was performed by a previously described method based on time-resolved fluorescence (Sjöroos et al. 1995). Sequence-specific oligonucleotide probes were used to identify the DQB1 alleles known to be significantly associated with either susceptibility to or protection against T1D in the Finnish population: DQB1*0302, DQB1*02, DQB1*0602, DQB1*0603, DQB1*0604 and DQB1*0301. The children were classified into four risk groups based on their HLA DQB1 genotype using the simplified classification presented in Table 1. (Ilonen et al. 1996) The high-risk and moderate-risk genotypes selected for follow-up have been observed to be present in 26.3% and 34.4% of Finnish children diagnosed with T1D before the age of 15 years, respectively, and among 2.3% and 9.9% of the background population (Nejentsev et al. 1999).

4.2.2 Autoantibody analyses

All the assays of diabetes-associated autoantibodies were performed in the Research Laboratory, Department of Pediatrics, University of Oulu.

ICA were quantified by a standard immunofluorescence method on sections of frozen human pancreas from a blood group O donor (Bottazzo et al. 1974) and detected with sheep fluorescein-conjugated anti-human IgG (Sigma, St.Louis, MO, USA). The end-point dilution titres of the ICA-positive samples were recorded and the results expressed in JDFU. The detection limit was 2.5 JDFU. Our research laboratory has participated in the international workshops on standardization of the ICA assay, in which its sensitivity was 100% and specificity 98% in the most relevant round (Greenbaum et al 1992). All samples initially positive for ICA were retested for confirmation.

Serum levels of IAA were quantified with a microassay modified from that described by Williams et al. (1997). Antibody-antigen complexes were precipitated with protein A Sepharose (Pharmacia Biotech, Uppsala, Sweden) after incubation of the serum samples

with mono-^{125}I-TyrA14-human insulin (Amersham, Little Chalfont, Bucks, UK) for 72 hours in the absence or presence of an excess of unlabelled insulin. The IAA titres representing specific binding were expressed in relative units (RU) based on a standard curve run on each plate using the MultiCalc^{TM} software program (PerkinElmer Life Sciences Wallac, Turku, Finland). A subject was considered positive for IAA when the specific binding exceeded 1.55 RU (99th percentile in 371 non-diabetic Finnish subjects). The disease sensitivity of the IAA microassay was 44% and its specificity 98% in the Center for Disease Control (CDC)-sponsored Diabetes Autoantibody Standardization Program (DASP) Workshop in 2002.

GADA were measured with a radiobinding assay, as described by Savola et al. (1998a). The recombinant plasmid encoding the whole GAD molecule was multiplied using E. coli cells and then purified. The TNT Coupled Reticulocyte Lysate System (Promega, Madison, Wi., USA) was used to transcribe and translate the GAD_{65} protein, which was labelled with ^{35}S-methionine (Amersham, Little Chalfont, Bucks, UK). Serum samples were incubated overnight with labelled protein, and protein A Sepharose (Pharmacia Biotech, Uppsala, Sweden) was used to isolate the immune complexes. After washing, the bound activity was measured in a liquid scintillation counter (1450 Microbeta Trilux, PerkinElmer Wallac, Turku, Finland). The results were expressed in relative units (RU) based on a standard curve constructed from the dilution of a pool of strongly positive samples with a negative sample. The cut-off limit for antibody positivity was set at the 99th percentile for 373 non-diabetic Finnish children and adolescents, i.e. 5.36 RU. The sensitivity of the GADA assay was 82% and its specificity 98% in the 2002 DASP Workshop.

IA-2A were quantified with a radiobinding assay, as described by Savola et al. (1998b). The recombinant plasmid encoding the intracellular portion of the IA-2 protein was multiplied using E. coli cells and then purified. The TNT Coupled Reticulocyte Lysate System (Promega, Madison, Wi., USA) was used to transcribe and translate the IA-2 protein, which was labelled with ^{35}S-methionine (Amersham, Little Chalfont, Bucks, UK). Serum samples were incubated overnight with labelled protein, and protein A Sepharose (Pharmacia Biotech, Uppsala, Sweden) was used to isolate the immune complexes. After washing, the bound activity was measured in a liquid scintillation counter (1450 Microbeta Trilux, PerkinElmer Wallac, Turku, Finland). Antibody titres were expressed in RU based on a standard curve, as for GADA. The cut-off limit for IA-2A positivity was 0.43 RU, which represents the 99th percentile in 374 healthy Finnish children and adolescents. The sensitivity of this assay was 62% and its specificity 100% in the 2002 DASP Workshop.

All samples with IAA, GADA or IA-2A levels between the 97th and 99.5th percentiles were reanalyzed to confirm their status. If there was a discrepancy between the two results, the sample was analyzed for a third time. A sample was considered positive if two out of three results were positive, and negative if two out of three results were negative. These quality control procedures meant that we retested about 2% of the initially negative samples (those between the 97th and 99th percentiles, the latter being the cut-off limit for autoantibody positivity) and more than 20% of the positive samples. The rate of false positives in the initial assay was less than 5% and the rate of false negatives less than 8%.

4.2.3 Statistical analyses

All the data in paper I were assessed based on the number of children remaining in the series at the time of the analyses. The pattern of autoantibody development and progression to T1D during the follow-up was deduced using Kaplan-Meier life-table survival analysis and log rank statistics. Sensitivity, specificity and positive predictive values (PPV) were calculated as described previously (Swets 1988). Cross-tabulation and Chi-square statistics were used to compare the frequencies of positivity, fluctuation and transient positivity between the various autoantibody reactivities. The time difference in the appearance of IAA between the high and moderate-risk children was evaluated with the t-test.

The distribution of autoantibodies between the risk groups was evaluated in paper II by cross-tabulation and Chi-square statistics with Yates correction, unless any expected value was less than five, when Fisher's exact test was applied. The t-test was used to assess age differences. The Kruskal-Wallis non-parametric analysis of variance and the Mann-Whitney U-test were used to compare antibody levels between the risk groups.

The analysis in paper III was based on the information generated by paper II, where it was shown that 4.2% of the children with an increased genetic risk tested positive for ICA and 2.3% for multiple antibodies. The probability of having more than one child in the family with ICA or with multiple autoantibodies was assessed from this data. The familial aggregation of ICA was tested using binomial probability calculations, from which a Chi-square value was derived. The differences in the distributions of the various genotypes were evaluated by cross-tabulation and Chi-square statistics. Time differences in the appearance of autoantibodies were assessed using the Kruskal-Wallis non-parametric test. When examining whether autoantibodies appear during the same season in siblings, the expected and observed frequencies were compared by cross-tabulation.

In order to account for the interval-censoring caused by the discrete time points of the measurements made in paper IV, the hazard ratios for seroconversion to positivity for T1D-associated autoantibodies during the 3-year follow-up period were estimated by life table survival regression in relation to region of residence, sex and genotype (Prentice and Gloeckler 1978). Two binary variables were used for region (Tampere vs. Turku and Oulu vs. Turku), and possible differences in the prevalence of the risk genotypes between the regions were sought by means of cross-tabulation and the Chi-square test.

A two-sided p value <0.05 was considered statistically significant in all the analyses.

5. Results

5.1 HLA DQB1 genotypes and the emergence of autoantibodies (I, II, III)

It could be seen clearly that an increased genetic risk of T1D defined by HLA DQB1 genotypes predisposed children to the appearance of disease-associated autoantibodies. The risk genotype was an independent risk factor, and increased risk could not be explained by the number of family members suffering from T1D. There were no sex differences in the distribution of the HLA DQB1 genotypes.

Among the 1,006 index cases, all of whose samples had been analyzed for ICA, IAA, GADA and IA-2A from birth, each antibody reactivity was more common in those with a high genetic risk than in those with a moderate risk when autoantibody positivity was defined as positivity in at least one sample ($p=0.034$ or less). If autoantibodies were required to be positive in at least two consecutive samples, the high-risk children had all antibodies except IA-2A more often than the moderate-risk children. 11/252 (4.4%) of the high-risk children and 16/754 (2.1%) of the moderate-risk children had IA-2A in at least two consecutive samples. Even though the high-risk children did not have IA-2A any more frequently than the moderate-risk children in this case, it could be seen that if IA-2A were to emerge, these antibodies appeared on average 6 months earlier in children with the high-risk genotype. This time difference was not significant, however ($p=0.64$). No such time difference in relation to the appearance of autoantibodies was seen for the other autoantibody reactivities in paper I. It was observed in paper II, focusing on the sib cohort, however, that ICA-positive subjects with the high-risk genotype were on average 2 years younger than those carrying the other genotypes ($p=0.04$), while no such age difference could be seen when comparing sibs positive for IAA, GADA or IA-2A who were carrying the high-risk genotype with the positive sibs having other genotypes.

The frequency of antibody positivity was also related to the genetic risk in the sib cohort, the sibs with genotypes conferring increased genetic risk being positive for various antibodies more often than those with decreased genetic risk (Table 4). Also, the frequency of multiple antibodies was related to the degree of genetic risk. Five of the 171 high-risk sibs (2.9%) tested positive for multiple antibodies (Fig. 2), and at least three antibodies could be detected in three high-risk sibs (1.8%) and six moderate-risk sibs (1.2%) but in none of those with genotypes conferring low or decreased risk (χ^2 for trends =12.61; $p=0.01$). One sib among those with the high-risk genotype (0.6%) and two among those with the moderate-risk genotypes (0.4%) tested positive for all four antibodies ($p=1.00$).

	I High risk	II Moderate risk	III Low risk	IV Decreased risk	Statistics
n	171	516	422	475	
ICA	11 (6.4); 3.3-11.2	18 (3.5); 2.1-5.5	7 (1.7); 0.7-3.4	6 (1.3); 0.5-2.7	χ^2=16.03;*P*=0.001 I vs. III *P*=0.005 I vs. IV *P*=0.001 II vs. IV *P*=0.04
IAA	4 (2.3); 0.6-5.9	9 (1.7); 0.8-3.3	1 (0.2); 0.01-1.3	3 (0.6); 0.1-1.8	χ^2=8.42; *P*=0.04 I vs. III *P*<0.05 II vs. III *P*<0.05
GADA	5 (2.9); 1.0-6.7	13 (2.5); 1.4-4.3	2 (0.5); 0.1-1.7	1 (0.2); 0.01-1.2	χ^2=15.82; *P*<0.001 I vs. III *P*=0.02 I vs. IV *P*=0.005 II vs. III *P*=0.02 II vs. IV *P*=0.002
IA-2A	3 (1.8); 0.4-5.0	6 (1.2); 0.4-2.5	1 (0.2); 0.01-1.3	0 (0); 0-0.8	χ^2=9.83; *P*=0.02 I vs. III *P*=0.02 II vs. IV *P*=0.03

TABLE 4. Frequencies of ICA, IAA, GADA and IA-2A in sibs with HLA DQB1 genotypes conferring differential degrees of genetic risk of T1D. Data are shown in absolute figures, frequencies (%) in brackets, and with 95% CI on the second line. Table 1 in paper II.

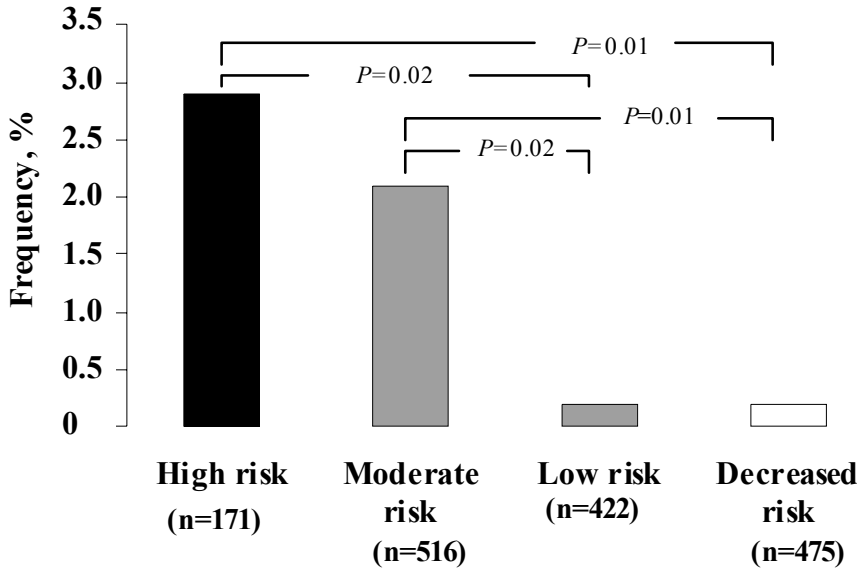

FIGURE 2. Frequency of multiple (> 2) autoantibodies among DIPP sibs with HLA DQB1 genotypes conferring differential degrees of genetic risk of T1D. Figure 1 in paper II.

The antibody levels also correlated with the risk genotypes. The ICA titres among the ICA-positive sibs were clearly related to the HLA-conferred disease susceptibility, with the highest titres recorded in those with the high-risk genotype and the lowest levels in those with genotypes conferring decreased risk. GADA levels were also related to genetic risk, in that the high-risk sibs had higher levels than the moderate-risk sibs, whereas IA-2A levels were higher in the moderate-risk sibs than in the high-risk ones (Table 5).

	I High risk	II Moderate risk	III Low risk	IV Decreased risk	Statistics
ICA, JDF units	18; 10-34	12.5; 7.5-29.5	10; 8-10	8; 5.8-8.5	χ^2=9.24; *P*=0.03 I vs. III *P*=0.03 I vs. IV *P*=0.03 II vs. IV *P*=0.05
IAA, RU	5.2; 2.5-13	6.6; 2.2-22.5	11.8*	4.8; 2.6-6.6	χ^2=0.24; *P*=0.89
GADA, RU	154.6; 91.4-181.3	9.3; 7.5-23.6	10#	181.8*	I vs. II *P*=0.01
IA-2A, RU	10.8; 8.7-79.4	115; 87.1-128.3	3.8*		I vs. II *P*=0.04

* only one positive subject, # only two positive subjects

TABLE 5. Levels of ICA, IAA, GADA and IA-2A in DIPP sibs with HLA DQB1 genotypes conferring differential degrees of genetic risk of T1D. Only children positive for the respective autoantibody were included in the analysis. Data are shown as medians with interquartile range. Table 2 in paper II.

In paper III 5.6% of the children (n=328) were positive for at least ICA on one occasion or more, and these carried the HLA DQB1 genotypes conferring increased disease susceptibility more often than did the other children. Also, the children with autoantibodies in families with at least two autoantibody-positive children (n=94) carried increased risk genotypes more frequently than all the other children (n=5742; p<0.001) or the children in families with a maximum of one child with at least ICA (n=5658; p<0.001) (Fig. 3). The ICA-positive siblings within the same family, i.e. families with a minimum of two autoantibody-positive children, had the same HLA DQB1 genotype (72.7%) more often than the siblings in families with only one or no child with autoantibodies (38.5%; p<0.001).

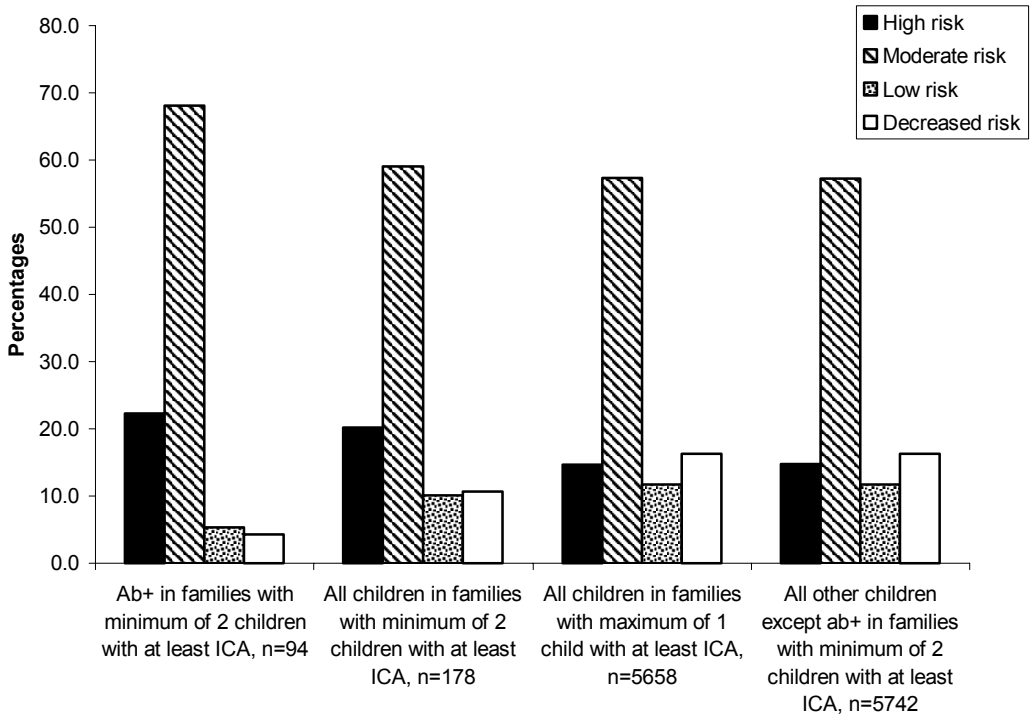

FIGURE 3. Distribution of HLA DQB1 genotypes in the children studied in paper III (ab+ = autoantibody positive children). (n=94 vs. n=5658 p<0.001 and n=94 vs. n=5742 p<0.001).

302 out of the 1,584 children in the sib cohort (19.1%) carried the protective DQB1*0602 allele, which is considered to be the strongest protective HLA class II allele, and none of them tested positive for IAA or IA-2A, whereas 17 (1.3%) of the other sibs had IAA (p=0.04) and 10 (0.8%) IA-2A (p=0.12). The sibs with the DQB1*0602 allele also tended to have ICA and GADA less often than did the other sibs [4 (1.3%) vs. 38 (3%); p=0.11, and 1 (0.3%) vs. 20 (1.6%); p=0.09]. None of the sibs with the DQB1*0602 allele had multiple ($\geq$ 2) autoantibodies.

5.2 Dynamics of autoantibodies (I, II, III, IV)

It was seen in paper I that all the antibody frequencies increased in the DIPP index cases steadily up to the age of 5 years, i.e. to the end of this analysis (Table 6). IAA were the predominant autoantibodies to appear. Altogether 139 out of the 1,006 children (13.8%) had at least one autoantibody at least once during the follow-up. Seventy-five (7.5%) had ICA at least once, 83 (8.3%) IAA, 46 (4.6%) GADA and 33 (3.3%) IA-2A (Fig. 4). If autoantibodies were required to be present in at least two consecutive samples, however, ICA were the most frequent to emerge (Fig. 5). Of the 44 children who had at least two autoantibodies, three had no ICA (6.8%), six no IAA (13.6%), 10 no GADA (22.7%) and

14 (31.8%) no IA-2A. Of the 95 children (9.4%) who had only one autoantibody, thirty-four (35.8%) had ICA, 45 (47.4%) IAA, 12 (12.6%) GADA, and 4 (4.2%) IA-2A.

Column number	I	II	III	IV	V		
Autoantibody type	≥1 abs at least once	≥1 abs in ≥ 2 consecutive samples	≥1 abs at least once	≥1 abs in ≥ 2 consecutive samples	≥1 abs persistently	*P* value I	*P* value II
ICA	75 (7.5)	67 (6.7)				0.488	
IAA	83 (8.3)	50 (5.0)				0.003	
GADA	46 (4.6)	34 (3.4)				0.171	
IA-2A	33 (3.3)	27 (2.7)				0.431	
At least one ab	139 (13.8)	91 (9.0)	105 (10.4)	61 (6.1)	67 (6.7)	<0.001	0.047
Multiple (≥2) abs	44 (4.4)	38 (3.8)	35 (3.5)	32 (3.2)	35 (3.5)	0.532	0.718

TABLE 6. Cumulative frequencies of autoantibodies (abs) by the age of 5 years in 1,006 DIPP index cases. All four autoantibodies (ICA, IAA, GADA, IA-2A) are considered in the first two columns and the fifth column and autoantibodies against biochemically characterized antigens (IAA, GADA, IA-2A) in the third and fourth columns. The P values I in the first four rows refer to a comparison of the frequencies in columns 1 and 2, whereas the values in rows 5 and 6 refer to a comparison between the first four columns. The P values II refer to a comparison between columns 2 and 5. Table 1 in paper I.

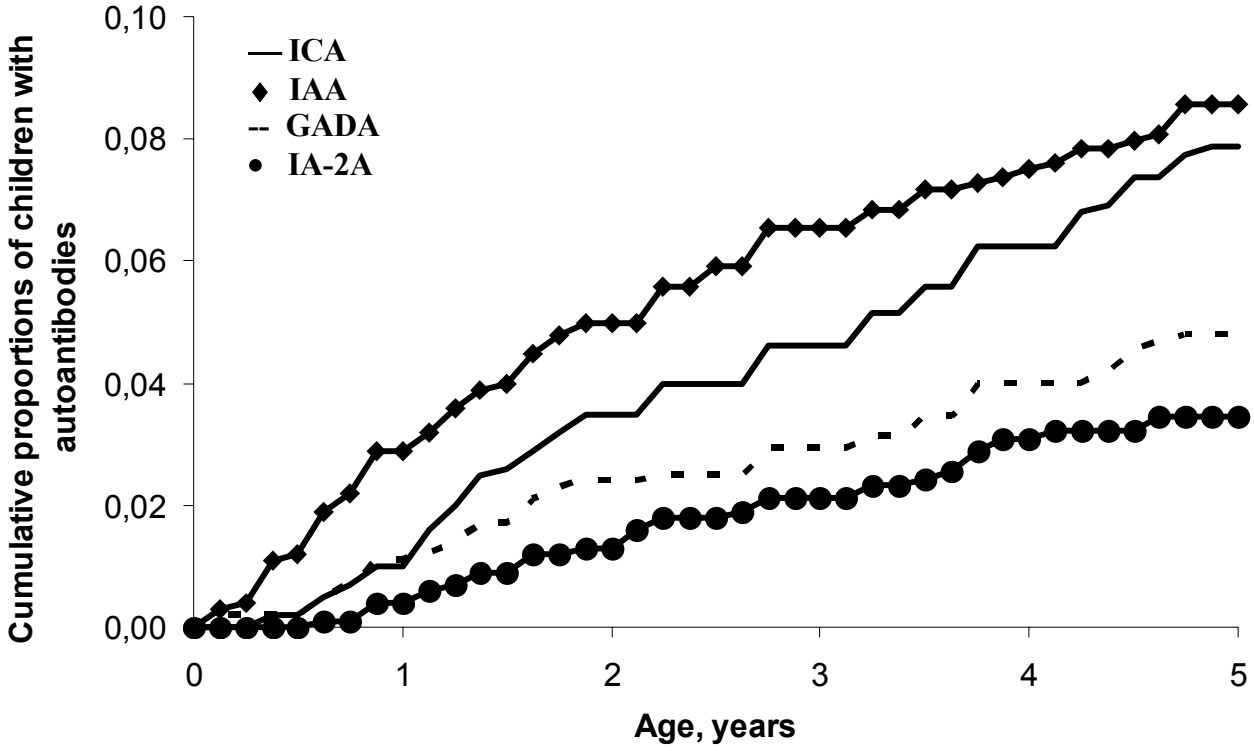

FIGURE 4. Development of autoantibodies by the age of 5 years in 1,006 DIPP index cases carrying increased HLA-conferred susceptibility to T1D, when antibody positivity was defined as positivity in at least one sample. Figure 1A in paper I.

FIGURE 5. Development of autoantibodies by the age of 5 years in 1,006 DIPP index cases carrying increased HLA-conferred susceptibility to T1D, when antibody positivity was defined as positivity in at least two consecutive samples. Figure 1B in paper I.

IAA were most often the first autoantibody reactivity to emerge in the 1,006 index cases, doing so in 65 cases (46.8%), ICA in 36 (25.9%), GADA in 19 (13.7%) and IA-2A in six cases (4.3%; IAA vs. the other autoantibodies $p<0.001$), while 13 (9.4%) already had multiple (≥2) autoantibodies in the first autoantibody-positive sample.

The life-table curves for children with at least two autoantibodies in paper I were essentially similar irrespective of whether a child was required to have autoantibodies in only one sample or in at least two consecutive samples (Fig. 6A). Omission of ICA from these analyses removed a large proportion of the children positive for only one autoantibody, whereas almost all the children with at least two autoantibodies remained (Fig. 6B). Among both the high-risk and moderate-risk children the omission of ICA removed a large proportion of the children positive for only one autoantibody, whereas almost all those with at least two autoantibodies could be identified on the basis of the biochemically defined autoantibodies (Fig. 7A and 7B).

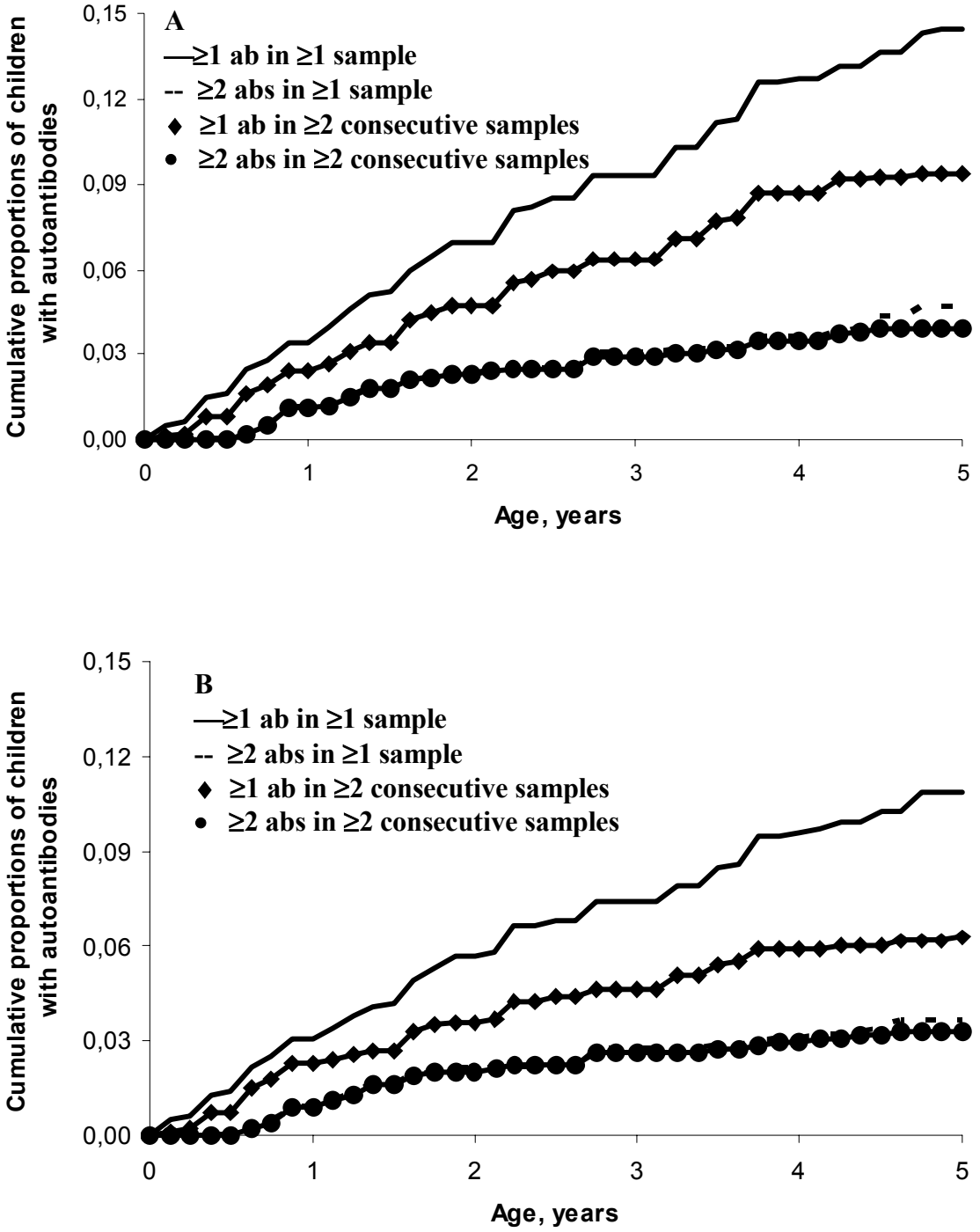

FIGURE 6. Development of autoantibodies (ab) when antibody positivity was defined as positivity for ICA, IAA, GADA and/or IA-2A (A), and development of autoantibodies when antibody positivity was defined as positivity for IAA, GADA and/or IA-2A (B) by the age of 5 years in 1,006 DIPP index cases carrying increased HLA-conferred susceptibility to T1D. Figure 2 in paper I.

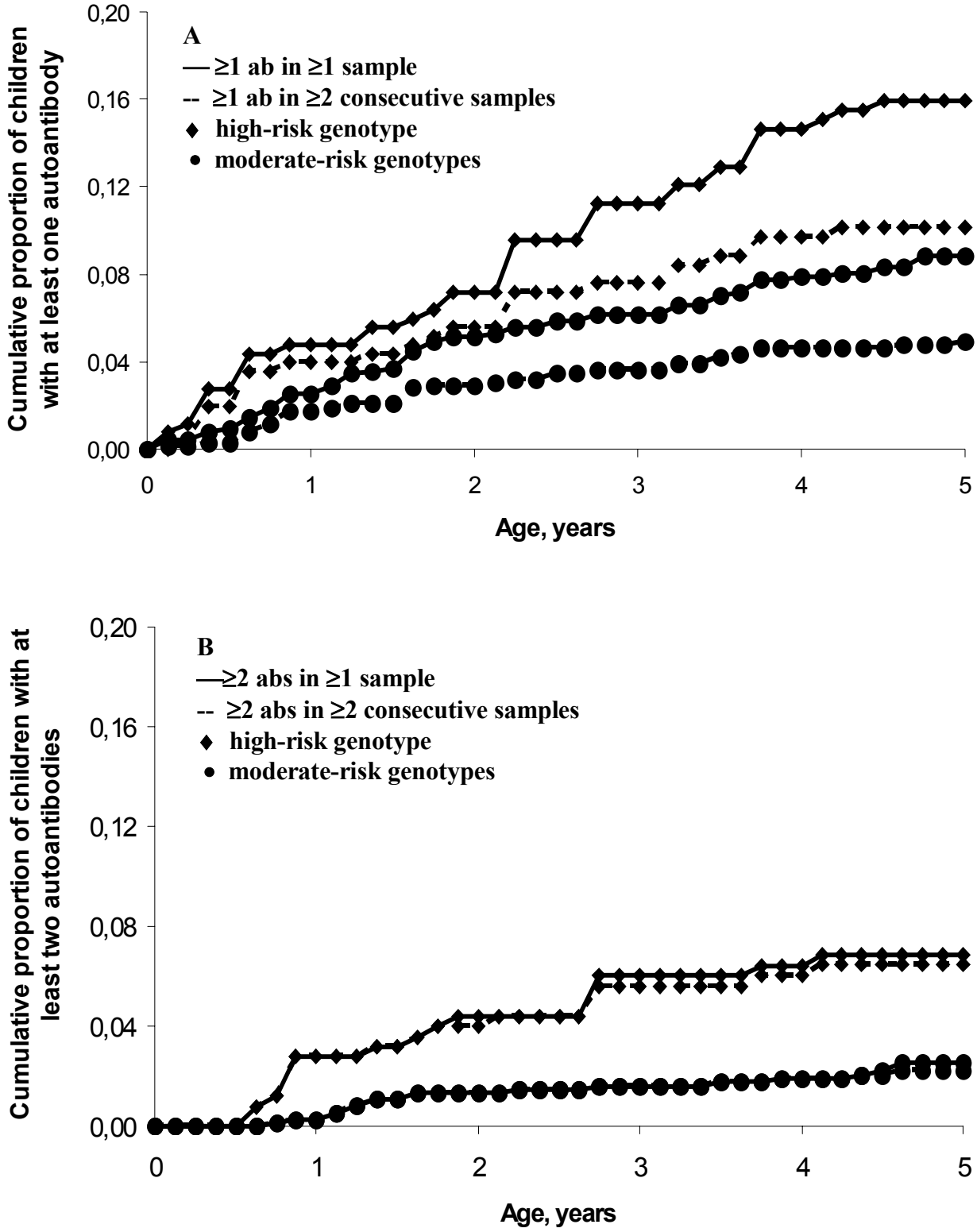

FIGURE 7. Development of autoantibodies when antibody positivity was defined as positivity for IAA, GADA and/or IA-2A by the age of 5 years in 1,006 DIPP index cases.

Comparison between high and moderate-risk genotypes: p<0.001 for at least one antibody in a minimum of one sample and p=0.003 for at least one antibody in a minimum of two consecutive samples; p=0.001 for at least two antibodies in both a minimum of one sample and a minimum of two samples. Figure 3 in paper I.

IAA were the most unstable of the four autoantibodies studied. None of the children in paper I had fluctuating ICA (more than one positive sample with one or more negative samples in between), whereas 1.1% had fluctuating IAA, 0.3% fluctuating GADA and 0.3% fluctuating IA-2A. The difference in the frequency of fluctuating antibodies was significant between ICA and IAA (p=0.001). Transiently positive ICA (one or more positive samples followed by at least two negative samples) were seen in 1.0% of the children, whereas 3.4% had transient IAA, 0.9% transient GADA and 0.6% transient IA-2A (IAA vs. ICA p<0.001; IAA vs. GADA p=0.021; IAA vs. IA-2A p=0.031). Since IAA had a fluctuating pattern most frequently, we examined whether the IAA titers had an effect on the rate of fluctuations or inverse seroconversions. The maximum IAA level was used for each child and the peak IAA titers were grouped into four quartiles. There were significantly less fluctuations and inverse seroconversions among those in the highest quartile than in the other three quartiles [highest (14.3%) vs. lowest (81.8%) p<0.001; highest vs. second quartile (81%) p<0.001; highest vs. third quartile (61.9%) p=0.001). There were no statistically significant differences between the other three quartiles in this respect.

There was an apparent relation between the number of detectable antibodies and ICA levels in paper II, children with four antibodies having significantly higher ICA levels (median 66 JDF U, interquartile range 34-258 JDF U) than those with two antibodies (16.5 JDF U, 8-34 JDF U; p=0.04) or with ICA only (8 JDF U, 6-10 JDF U; p<0.01). The ICA level was also significantly higher in those with three antibodies (23 JDF U, 13.8-55 JDF U) than in those with one antibody (p<0.01) and higher in those with two antibodies than in those with only one (p=0.05).

When the sibs were divided into two age groups (<5 and ≥5 years) in paper II, 2.0% (15/734) of the younger subjects and 3.2% (27/850; p=0.16) of the older ones tested positive for ICA. No differences were observed between these two age groups in the frequencies or levels of the other antibodies.

There were no significant differences in the frequencies or levels of the various antibodies between the sexes. With regard to season, IAA tended to appear less often during the summer than during any other season in paper III and GADA more often in the spring than at any other time (Fig. 8).

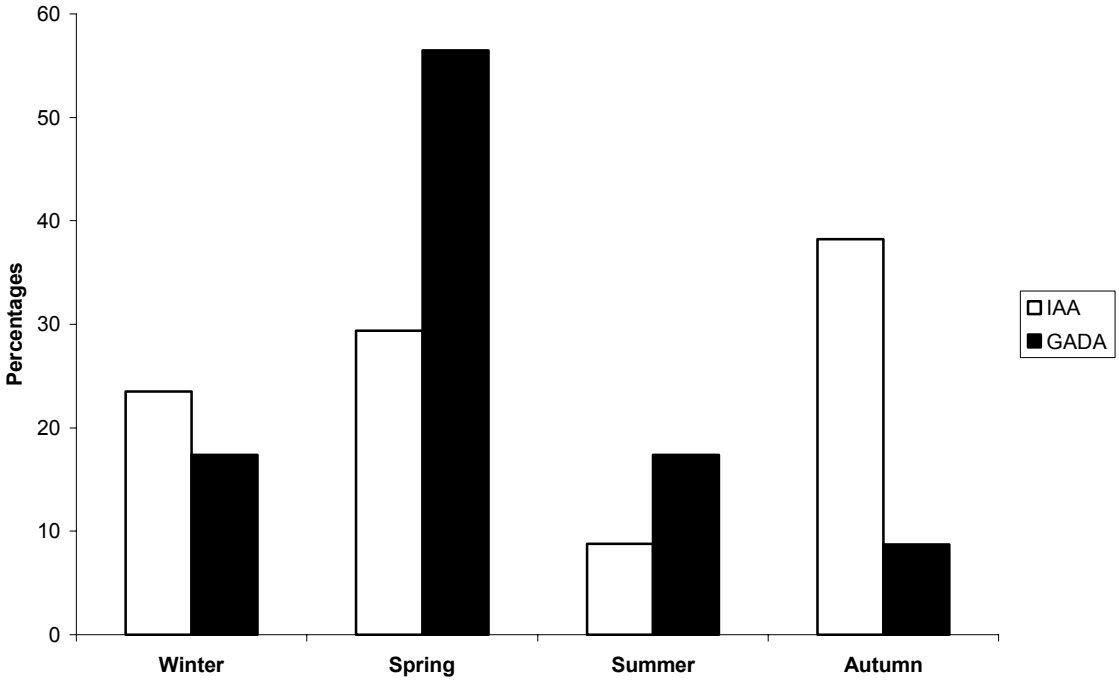

FIGURE 8. Seasonal timing of the appearance of IAA and GADA in children of families with at least two autoantibody positive siblings in paper III. IAA tended to appear less frequently than expected during the summer (p=0.15) and GADA more often than expected in the spring (p=0.06). (winter=December, January, February; spring=March, April, May; summer=June, July, August; and autumn=September, October, November. Figure 3 in paper III)

5.3 Aggregation of beta-cell autoimmunity (III, IV)

A clear familial aggregation of autoantibodies was observed in paper III. There were altogether 328 of 5836 (5.6%) children who were positive for ICA at least once, and 94 of these belonged to the 44 (1.9%) families with at least two autoantibody-positive children (out of the total of 2283 families). This represents an obvious familial aggregation of ICA positivity. Likewise, while the expected number of families with two ICA-positive children in this population was 9.8 (0.4%), the observed proportion was close to five times higher than this ($p<0.001$). In addition, the frequency of multiple (≥ 2) autoantibodies showed familial aggregation, the number of families with two children positive for multiple autoantibodies being 9 (0.39%), whereas the expected number was 3 (0.13%). Thus the observed number was three times the expected one ($p<0.001$).

The autoantibody-positive children within the same family did not usually seroconvert to autoantibody-positivity at the same time (Table 7). The shortest median time interval between siblings was observed for GADA (0.2 years, range 0.0-1.6) and the longest for

IA-2A (2.5 years, range 1.8-3.2). Furthermore, siblings rarely seroconverted to autoantibody-positivity at the same age (Table 7). The smallest median age difference was 3.3 years, for ICA (range 0.0-10.5 years), and the most conspicuous 9.6 years, for IA-2A (range 0.4-12.6 years). In only 27.3% of the families with at least two autoantibody positive children did those with antibodies have exactly the same autoantibody/autoantibodies. IAA and the first autoantibody (Fig. 9), which could be any of the four markers studied here, appeared in the sibling pairs during the same season more often than would be expected on the assumption of an even distribution between the seasons ($p<0.001$ and $p=0.018$, respectively).

	Median time-lag (range)	Median age difference (range)
ICA (n=17)	1.6 (0.0-3.2)	3.3 (0.0-10.5)
IAA (n=12)	1.4 (0.0-4.4)	4.0 (1.4-8.6)
GADA (n=5)	0.2 (0.0-1.6)	6.6 (2.3-14.2)
IA-2A (n=3)	2.5 (1.8-3.2)	9.6 (0.4-12.6)
First autoantibody (n=41)	1.0 (0.0-5.8)	4.3 (0.0-14.7)

n = number of sib-pairs

TABLE 7. Time intervals (in years) between the first autoantibody-positive samples of siblings within the same DIPP family and differences in age (in years) at seroconversion to autoantibody positivity among antibody-positive siblings within the same family provided that the timing of the seroconversion was known. Table 1 in paper III.

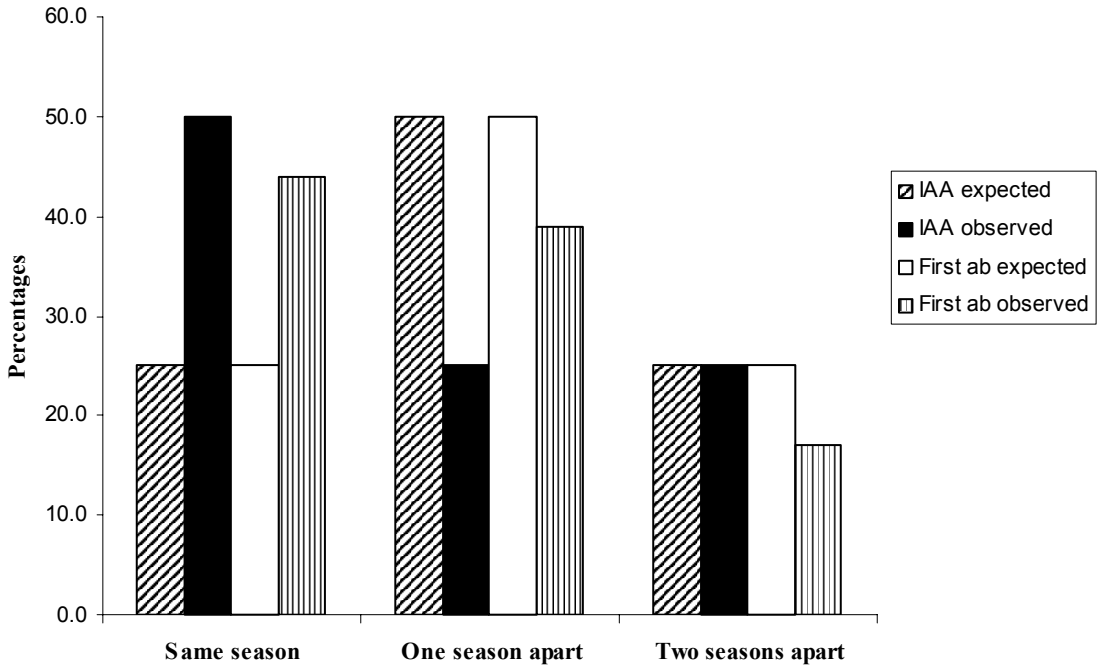

FIGURE 9. Expected and observed distributions of the appearance of IAA and the first autoantibody (any of the four antibodies studied here) by season in siblings in the same family. Both IAA and the first autoantibody appeared in the sibling pairs during the same season more often than was expected (P<0.001 and P=0.018, respectively). Figure 2 in paper III.

The analysis of regional variations in beta-cell autoimmunity among the DIPP index cases in paper IV showed that the high and moderate-risk genotypes were unevenly distributed between the three university hospital regions of Turku, Oulu and Tampere ($p<0.001$), the Turku and Tampere regions having similar proportions of the high and moderate-risk genotypes ($p=1.00$), whereas Oulu had a higher proportion of children with moderate-risk genotypes and less with the high-risk genotype than either Turku ($p<0.001$) or Tampere ($p<0.001$) (Fig. 10).

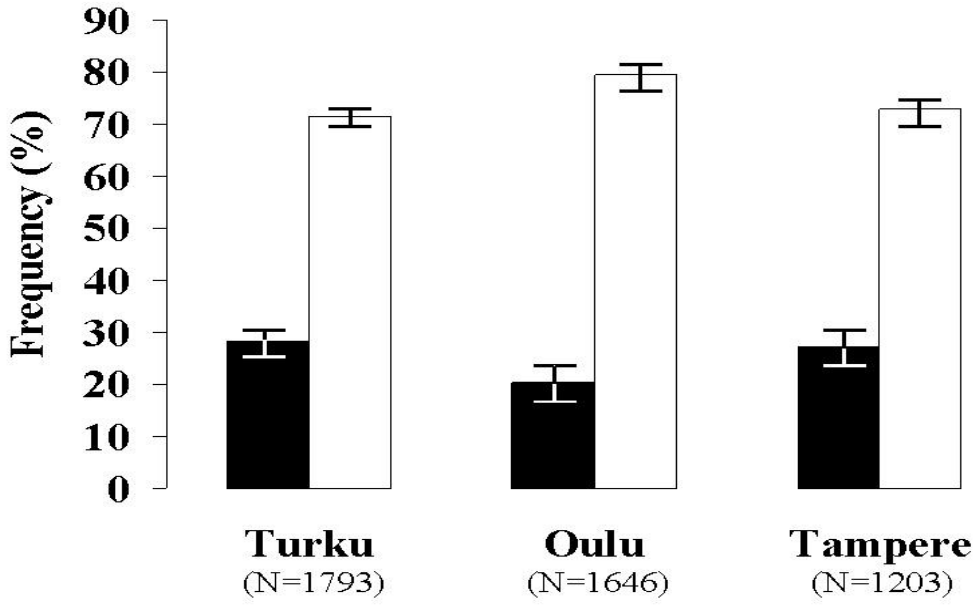

FIGURE 10. Prevalences of high (black columns) and moderate-risk (open columns) genotypes among DIPP index cases in the Turku, Oulu and Tampere regions. The bars represent the 95% confidence intervals. Figure 2 in paper IV.

Residence in the Tampere region was associated with a reduced rate of seroconversion to ICA positivity, a difference that remained significant even when the HLA DQB1 genotype was taken into account (Table 8A). There were significant differences between Tampere and Turku among those with the moderate-risk genotypes (Fig. 11). A conspicuous feature was that not a single high-risk subject seroconverted to ICA positivity during the first year of life in Tampere. Positivity for multiple autoantibodies, i.e. ICA and at least one other autoantibody, was also less common in Tampere (Table 8B). The differences between the two regions could be seen clearly among those with the high-risk genotype (p=0.025), whereas there were no regional differences between the children carrying moderate-risk genotypes (p=0.40). Almost 4% of the total cohort (171/4,642; 3.7%) had at least one family member affected by T1D, comprising 74, 48, and 49 children in Turku, Oulu and Tampere, respectively (4.1%, 2.9%, and 4.1%; p=0.12).

A

	Unadjusted HR	95% CI	p-value	Adjusted * HR	95% CI	p-value
Region						
Tampere vs. Turku	0.53	0.32-0.86	0.008	0.54	0.32-0.87	0.010
Oulu vs. Turku	0.70	0.49-0.99	0.044	0.74	0.51-1.05	0.092
Genotype						
High vs. moderate risk	1.94	1.39-2.68	<0.001	1.90	1.36-2.59	<0.001

B

Region						
Tampere vs. Turku	0.42	0.17-0.88	0.021	0.42	0.17-0.90	0.024
Oulu vs. Turku	0.83	0.49-1.38	0.471	0.92	0.54-1.53	0.738
Genotype						
High vs. moderate risk				2.89	1.78-4.69	<0.001

*Adjusted for all the variables in the table.

TABLE 8. Hazard ratios (HR) and 95% confidence intervals (CI) for seroconversion to ICA positivity (A) and the possession of at least one other autoantibody in addition to ICA (B) associated with living in the regions of the Turku, Oulu, or Tampere University Hospitals, and in association with HLA DQB1 genotype. Table 2 in paper IV.

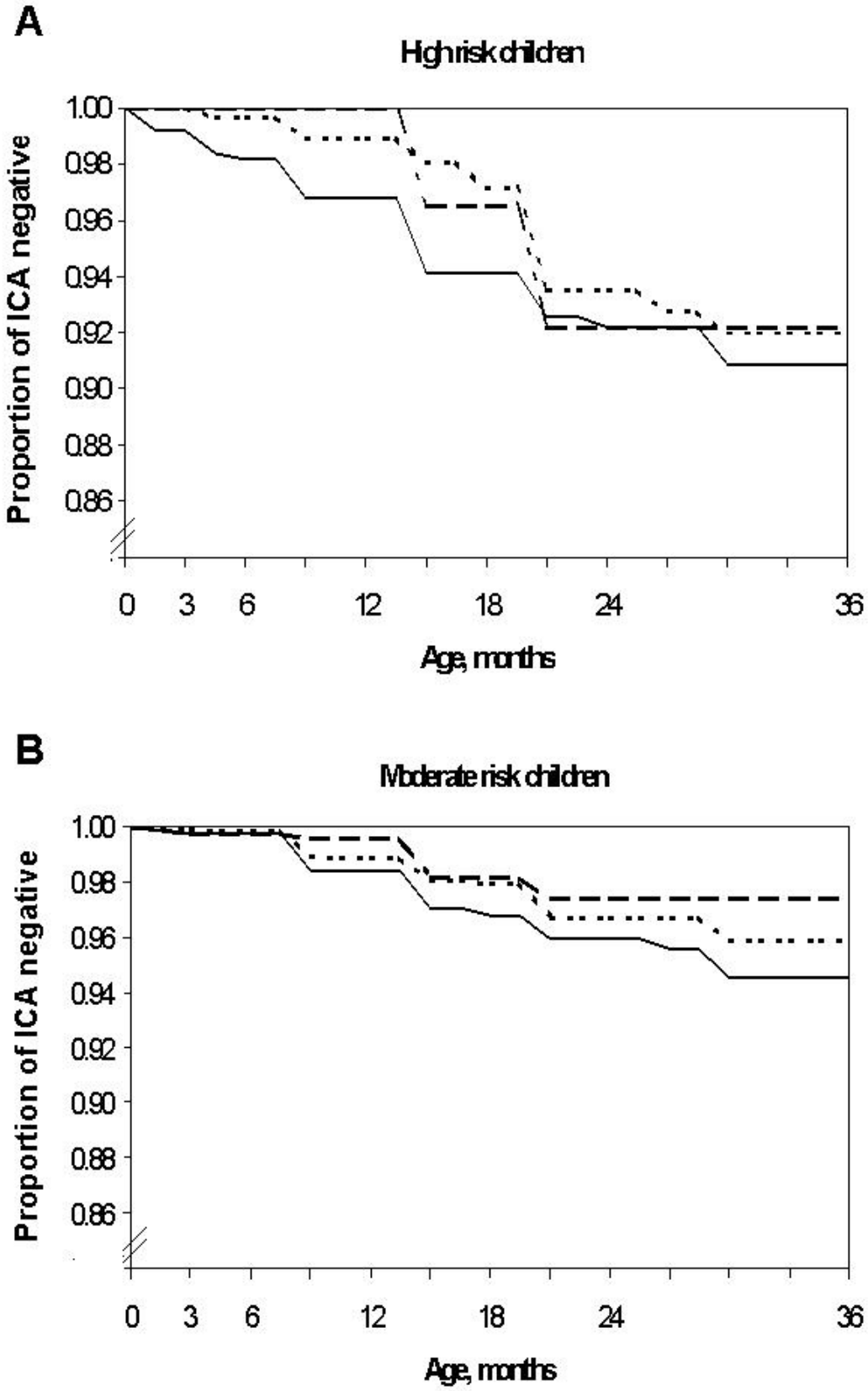
A
High risk children
Proportion of ICA negative
1.00
0.98
0.96
0.94
0.92
0.90
0.88
0.86
0
3
6
12
18
24
36
Age, months
B
Moderate risk children
Proportion of ICA negative
1.00
0.98
0.96
0.94
0.92
0.90
0.88
0.86
0
3
6
12
18
24
36
Age, months

*FIGURE 11. Appearance of ICA in the DIPP index cases who were heterozygous for HLA DQB1*02/0302 (A) or carried the HLA DQB1*0302/x (x¹*02, *0301, *0602, *0603-4) genotype (B) in Turku (———), Oulu (·····), and Tampere (-----). The hazard ratio (95% confidence interval) for seroconversion to ICA positivity associated with living in Tampere vs. Turku was 0.62 (0.28-1.25) among the children carrying the high-risk genotype and 0.49 (0.24-0.92) among those with moderate-risk genotypes. Figure 3 in paper IV.*

5.4 Type 1 diabetes, HLA DQB1 risk genotypes and autoantibodies (I, II, IV)

Thirteen subjects (1.3%) in the series enrolled for paper I had developed T1D by the age of 5 years, all of them having developed at least two autoantibodies before clinical disease. Genetically high-risk children developed the disease more often than moderate-risk ones, since 3.6% (9/252) progressed to T1D as opposed to only 0.5% (4/754) of the latter (Fig. 12; $p<0.001$).

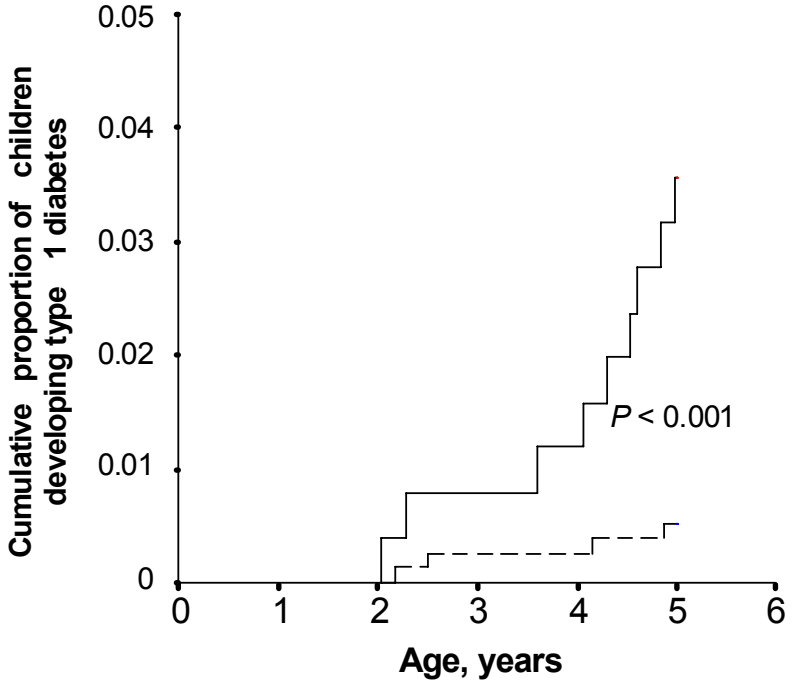

FIGURE 12. Development of T1D among 1,006 DIPP index cases, analysed separately for those with the high-risk HLA genotype (———) and those with moderate-risk genotypes (-----). Figure 4 in paper I.

We also studied the predictive characteristics of autoantibodies among the 1,006 index cases monitored from birth. The most sensitive predictors of T1D were ICA and IAA, while the most specific predictor was IA-2A, which also had the best positive predictive value for clinical T1D (Table 9), although the confidence intervals overlapped due to the limited number of progressors.

Autoantibody positivity	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)
ICA	100 (75-100)	94 (92-95)	17 (10-28)
IAA	100 (75-100)	93 (91-95)	16 (9-25)
GADA	85 (55-98)	96 (95-98)	24 (13-39)
IA-2A	69 (39-91)	98 (96-98)	27 (13-46)
≥ 2 antibodies	100 (75-100)	97 (96-98)	30 (17-45)
≥ 2 antibodies out of IAA, GADA and IA-2A	92 (64-100)	98 (97-99)	34 (19-52)

TABLE 9. Sensitivity, specificity and positive predictive value (PPV) of autoantibodies and their combinations for progression to clinical T1D (n=13) by the age of 5 years among 1,006 DIPP index cases. Table 2 in paper I.

The children who had at least one affected family member developed autoantibodies more often than those without familial diabetes. In paper IV 7.0% (12/171) of the index cases with an affected family member tested positive for ICA at least, while 3.1% (138/4471) of the other children had ICA at least (p=0.004). A higher proportion of the 38 sibs in paper II with at least one first-degree relative affected by T1D tested positive for IA-2A (2; 5.3% vs. 8; 0.6% p=0.02) and multiple antibodies (3; 7.9% vs.15; 1.0%; p=0.008) than of the sibs without affected family members.

6 Discussion

6.1 Study population

The population evaluated in this thesis was unique even from a global perspective. T1D has been widely studied in first-degree relatives of patients with the same disease, in spite of the fact that only approximately 10% of patients with newly diagnosed T1D have an affected family member, the rest of the cases being sporadic. For this reason it is important to study the disease process in the general population, and this is what the Finnish DIPP study set out to do. Another survey partly based on the general population is the Diabetes Autoimmunity Study in the Young (DAISY) in Colorado, USA, which also identifies study children at birth by genetic screening (Rewers et al. 1996). The group of children observed is substantially smaller, however, and the rates of seroconversion to autoantibody positivity and progression to overt T1D in Colorado are less than a third of those in Finland.

The Finnish people have a positive attitude towards research, and the health care infrastructure is well organized and provides a good basis for observational studies such as DIPP. The high rate of parental consent to the genetic screening of their offspring for HLA-conferred T1D risk, about 94%, reflects this positive attitude and means that the index cases are well representive of the general population in Finland. Kupila et al. (2001) have already earlier reported that population-based screening for T1D risk genotypes is widely accepted in Finland and adherence to long-term follow-up regimes is high. They also conclude that the selection of susceptibility and protective HLA DQB1 alleles used in DIPP works well for the prediction of early-onset T1D at least.

The proportion of children with a HLA-defined predisposition to T1D in paper II, i.e. those with high or moderate-risk genotypes, was considerably greater among the sibs (43.4%) than among the index cases in the DIPP study (about 14%). This shows that the risk genotypes are enriched in this series relative to an unselected general population cohort, due to the fact that these children are sibs of individuals who carry high or moderate-risk genotypes. This only adds to the power of the present research, however, since the proportion of cases with enhanced genetic disease susceptibility is tripled relative to a completely unselected population.

Paper IV focused on possible regional differences in HLA genotypes conferring susceptibility to T1D and in the frequency of autoantibody seroconversion, and accordingly the data were handled separately for each region, whereas in the DIPP study in general the three regions are combined to form a single data set.

The DIPP study includes a randomized, controlled, double-blinded intervention with daily administration of intranasal insulin for genetically susceptible children testing positive for multiple autoantibodies (i.e. ICA + at least one other antibody) in a minimum of two consecutive samples. In most cases the intervention trial has no effect on the observations reported here, since the end-points are positivity for ICA alone or combined with at least one other autoantibody reactivity, the latter end-point being identical to the inclusion criterion for the intervention trial. The trial may have some impact on the reported predictive characteristics of various autoantibodies in the series of 1,006 index

cases observed for all four autoantibodies from birth, since 9 of the 13 progressors had been assigned to it.

In conclusion, the DIPP study is a large population-based survey with a high participation rate and low numbers of drop-outs, since 76% of the children at risk attended for the 48-month visit (Kupila et al. 2001). The HLA DQB1 typing was performed in Turku, and was subjected to continuous quality control assessment, and the autoantibody analyses were carried out at one central laboratory to eliminate inter-laboratory differences. The DIPP core laboratories are involved in international quality control and standardization activities to maintain the high-quality of their autoantibody assays.

6.2 HLA DQB1 genotypes and the development of autoantibodies

Although the Finnish population is considered to be fairly homogeneous and has been relatively isolated for many centuries (Nevanlinna 1972), there are some geographical differences in the prevalences of HLA genotypes and alleles within the country. The National Bone Marrow Register has also been used to study HLA allele frequencies in the Finnish population, and substantial regional variations have been perceived in allele frequencies (Siren et al. 1996). A difference in T1D-associated HLA DQB1 genotypes has been reported earlier between the south-western part of the country (Turku) and the northern part (Oulu) based on the DIPP cohort (Ilonen et al. 2000), and the present work expanded the number of subjects and regions, as Tampere was also included in the analysis. The present findings confirm the previous observation that the proportion of high-risk children is significantly lower in the Oulu region than in the Turku region but also lower than in the Tampere region.

As hypothesized, all the analyses showed that HLA DQB1 risk genotypes are powerful determinants of beta-cell autoimmunity in non-diabetic children derived from the general population. The results indicated that the high and moderate-risk genotypes predispose carriers to the emergence of diabetes-associated antibodies, and that the low and decreased-risk genotypes provide protection from such beta-cell autoimmunity. Among the HLA DQB1 genotypes conferring increased risk, the high-risk genotype enhanced the risk of appearance of autoantibodies even more conspicuously than did the moderate-risk genotypes and had an effect on autoantibodies at several levels. It had an influence on the appearance of single autoantibody specificities, on the appearance of multiple autoantibodies, and on autoantibody levels. This suggests that the HLA DQB1 genotype affected both the quantity and quality of beta-cell autoimmunity. This close association between HLA DQB1 genotypes and the appearance of autoantibodies implies that the genotype screening program has been designed in an effective way with a view to identifying optimal candidates for immunological monitoring, i.e. the subjects who will be most likely to benefit from any preventive treatment, assuming that effective measures can be identified.

The present findings show that the HLA DQB1 *0602 allele protects subjects from the appearance of multiple antibodies, although there are still a few individuals with this protective allele who test positive for single antibodies. This could be interpreted as

indicating that individuals with this allele may show signs of beta-cell autoimmunity, but it is not progressive and may fade away over time.

The HLA DQB1 genotype appeared to accelerate the emergence of autoantibodies to some extent, as it was seen in paper I that IA-2A tended to appear earlier in the high-risk children than in the moderate-risk ones and in paper II that the ICA-positive subjects with the high-risk genotype were on an average 2 years younger than those carrying the other genotypes. Previous authors have suggested that IA-2A may be related to rapid progression to T1D (Christie et al. 1994, Gardner et al. 1999), and a recent report has indicated that IA-2A positivity is a more direct predictor of progression to overt diabetes in siblings of patients with T1D than positivity for multiple autoantibodies *per se* (Decochez et al. 2002). The tendency for IA-2A to appear earlier in high-risk children could imply more aggressive development of T1D than in moderate-risk children, and would require more immediate intervention in the preclinical phase of T1D.

6.3 Dynamics of humoral autoimmunity in preclinical type 1 diabetes

Although the frequency of various diabetes-associated autoantibodies increased at a relatively stable rate at least up to the age of 5 years, no significant differences in the frequencies of the various antibodies could be detected between the sibs under 5 years of age and those aged 5 years or older. This implies that the majority of susceptible children who are to seroconvert to antibody positivity do so before the age of 5 years. Earlier reports also support the view that beta-cell autoimmunity both in the offspring of parents with T1D (Ziegler et al. 1999) and in the general population (Kimpimäki et al. 2001) may be initiated early in life. Also, the fact that antibody levels were of the same magnitude in the younger and older age groups may suggest that the levels remain relatively stable for a reasonable time after seroconversion, and that those who develop high antibody titres already have these at an early stage. An alternative explanation is that similar proportions of children have diminishing and rising antibody levels as they get older. The general implication is, however, that the screening of susceptible children, e.g. for preventive trials, can and should be initiated at an early age. Susceptible individuals can be identified at an early age, and possible future preventive measures might be more effective if started early in the disease process.

It has been shown previously that multiple autoantibodies can be used as surrogate markers of a high risk of T1D in young children, whereas the presence of single autoantibody reactivity may represent innocent, non-progressive beta-cell autoimmunity (Kimpimäki et al. 2000) or a very early stage of progressive autoimmunity. We set out here to evaluate how stable multiple autoantibody positivity is by comparison with single autoantibody positivity. When children with antibodies in at least one sample or in at least two consecutive samples were compared, it could be observed that in these two groups the proportions of autoantibody-positive subjects differed only slightly among those with at least two autoantibodies but more conspicuously among those with a minimum of one autoantibody, which could be interpreted as implying that when multiple antibodies appear they are more permanent, while single autoantibody positivity can more easily fluctuate and disappear, thus reflecting non-destructive autoimmunity. We also set out to determine whether there was any relation between the number of

antibodies and the level of ICA in those who tested positive for ICA, since both are measures of the aggressiveness of beta-cell autoimmunity and increase as the intensity of the beta-cell immunity increases. The results confirmed such an association, as ICA levels acted in concert with the number of antibodies: the higher the level of ICA, the higher the number of antibodies. This autoantibody pattern supports the view that persistent positivity for two or more autoantibodies reflects destructive progressive beta-cell autoimmunity, whereas positivity for a single autoantibody represents harmless non-progressive or even regressive beta-cell autoimmunity.

When children with at least one autoantibody in at least one follow-up sample were regarded as autoantibody-positive, the most common diabetes-associated autoantibodies to appear were IAA, but when at least one autoantibody was required to be present in a minimum of two consecutive samples, ICA emerged more commonly than IAA. This agrees with the observation that IAA show more fluctuations and inverse seroconversions than ICA. It was also evident, however, that autoantibody levels had an effect on the stability of autoantibody status, in that the children who developed high-titre IAA were more likely to remain IAA-positive than those with lower levels. By raising the cut-off limit for IAA positivity, the proportion of children with fluctuations and inverse seroconversions could be reduced, but the sensitivity of IAA for the identification of subjects with signs of multiple autoantibody positivity would decrease correspondingly.

6.4 Familial and geographical clustering of beta-cell autoimmunity

One of our aims was to study whether beta-cell autoimmunity shows familial aggregation, as does the clinical disease. The risk of T1D in siblings of diabetic probands up to the age of 30 years has been estimated to be approximately 6-7% (Wagner et al. 1982, Tarn et al. 1988, Gavard et al. 1992, Lorenzen et al. 1994), exceeding the risk in the background population by 10-20 times. In our cohort of children with increased HLA-conferred susceptibility to T1D and their siblings derived from the general population we saw a clear familial aggregation of beta-cell autoimmunity. The fact that the series included a substantial proportion of children with HLA DQB1 genotypes other than those conferring increased genetic susceptibility to T1D implies that the expected number of 9.8 families with two ICA-positive children based on our sibling cohort represents an overestimation of the expected frequency rather than an underestimation, and therefore this accumulation is likely in reality to be even more pronounced.

A sib's risk of developing ICA was almost five times higher than expected and the risk of developing multiple autoantibodies three times higher. As mentioned above, the risk of developing T1D is usually stated to be at least 10 times higher in siblings of diabetic patients than in the background population. Accordingly this relative risk is higher than that of developing diabetes-associated autoantibodies observed in the present survey. One explanation might be that the children in this series were very young, with a median initial age of 2.2 years. As noted above, it seems that the frequency of autoantibodies increases steadily at least up to the age of 5 years, and therefore this aggregation of autoimmunity might still increase with time. At least in relation to clinical T1D, it has been shown that clustering increases over time (Pociot et al. 1993).

It may be assumed that the environmental factors involved in the development of beta-cell autoimmunity and overt T1D, e.g. dietary factors, infections and living conditions, should be the same in siblings, but even so, autoantibodies rarely appeared at the same time or at the same age in seroconverting siblings belonging to the same family, i.e. the duration of beta-cell autoimmunity varied from one child to another within the family. This discrepancy suggests either that the induction of beta-cell autoimmunity is modified by host-related factors or that the exogenous factors triggering beta-cell autoimmunity are different even in members of the same family. One host-related consideration could be some genetic factor or factors other than HLA DQB1 alleles, which might promote or delay the appearance of autoimmunity in one of the siblings.

Although IAA did not emerge at the same time in the siblings, they appeared during the same season within a family more often than would have been expected, as also did the first autoantibody to emerge. The observation that the first autoantibody seems to emerge during the same season in siblings may indicate that the triggering determinant is related to a factor or factors with seasonal variation, and the finding that IAA and GADA show seasonal variation in their appearance as well points in the same direction. Such factors may be infectious agents, e.g. enterovirus infections, which peak in late summer with another smaller peak in late winter (Tauriainen et al. 2003). The fall IAA peak fits well into this pattern, especially since IAA are usually the first or among the first autoantibodies to appear in young children (Ziegler et al. 1999, Kimpimäki et al. 2001).

The identification of families having multiple members with signs of beta-cell autoimmunity may provide a valuable population for studies on the pathogenetic process leading to clinical T1D. As the living conditions of siblings are largely the same, it will be interesting to see later which children in this population will progress to clinical T1D and what factors differentiate them from their non-progressing siblings.

Our data demonstrate that T1D-associated autoantibodies show geographical aggregation that cannot be explained by HLA DQB1 risk genotypes or the proportion of first-degree relatives affected by T1D. There was a clear regional difference in the risk of seroconversion to ICA positivity, this being highest in the Turku region and lowest in the Tampere area. The reduced frequency of ICA positivity observed in the Oulu region relative to Turku became non-significant after adjustment for HLA genotypes. Since positivity for multiple autoantibodies is linked to a markedly increased risk of progression to clinical disease (Bingley et al. 19949), it was of interest to assess whether the incidence of multiple autoantibodies differed between the three regions. These analyses gave similar results to those observed for ICA, as the children in the Tampere region also had multiple autoantibodies less often than age-matched children in the Turku area. Since the presence of multiple autoantibodies is considered to be a sign of destructive beta-cell autoimmunity and therefore a predictive marker of clinical disease, the protective effect of residence in Tampere or the predisposing effect of residence in Turku with regard to the appearance of autoantibodies could suggest that the risk of developing overt T1D is also less in the Tampere region. This question will be addressed after a longer follow-up of the population, employing similar analyses to those performed here and using clinical T1D as the outcome. Such an assessment is clearly indicated in the light of the current observations.

The regional difference in the appearance of signs of beta-cell autoimmunity also offers a fruitful setting for examining environmental differences between the regions, since this finding could be explained by environmental factors. Another explanation might be that allele frequencies in other loci conferring susceptibility to T1D, either within or outside the HLA region of chromosome 6, may differ between the regions.

6.5 ICA for screening of beta-cell autoimmunity

ICA were used as the screening tool in the immunological follow-up of the DIPP study since they constituted the best validated marker of beta-cell autoimmunity at the time when the study was started. They were also the best described indicator of on-going beta-cell damage and their predictive value had been observed to be high in first-degree relatives of children with diabetes (Karjalainen 1990, Karjalainen et al. 1996). In the present work the utility of ICA as a primary screening tool for beta-cell autoimmunity was tested in a large series based on the general population and compared with the role of autoantibodies to biochemically characterized antigens (IAA, GADA, and IA-2A).

The positive predictive values of ICA, IAA, GADA, and IA-2A in siblings of children with T1D in the Finnish DiMe study (Kulmala et al. 1998) were 43, 29, 42 and 55%, and their sensitivities 81, 25, 69 and 69%, respectively, indicating that ICA have the highest disease sensitivity. Although such a high sensitivity is most likely to lead to the detection of some harmless autoimmunity that will fade away, ICA testing provides a population with a risk of progression to clinical T1D on which it is possible to conduct further autoantibody and metabolic testing in order to identify the individuals at the highest risk of progression and take potential preventive measures.

When comparing ICA with autoantibodies to biochemically characterized autoantigens, we found that a significant proportion of the children with one autoantibody would have been missed without ICA, but that the proportion of children with multiple autoantibodies would have altered only marginally. It can thus be seen that ICA also identify a substantial number of children who develop only ICA and are less likely to present with clinical T1D at any time. It is possible that isolated ICA positivity may represent a phase of harmless beta-cell autoimmunity that may later develop into destructive beta-cell autoimmunity in some individuals, as reflected by the appearance of other diabetes-associated autoantibodies (Knip 1997). Isolated ICA positivity may also persist as an innocuous epiphenomenon, or fade away, as inverse seroconversions have been seen during the long-term observation of initially ICA-positive children (Savola et al. 2001). Although the overwhelming majority of children who had at least two autoantibody reactivities were identified as autoantibody-positive without ICA, ICA were the most common single autoantibody in the children with multiple autoantibodies. This illustrates the fact that ICA were more sensitive in our hands than the other three autoantibodies for identifying young children who will develop multiple autoantibodies and who have a marked risk of developing clinical T1D.

The ICA assay appears to work well in young children, since the ICA-positive children in the high-risk group of the sib cohort were younger than those in the other risk groups.

This implies that children with the high-risk genotype tend to seroconvert to ICA positivity at a younger age, and that the DQB1*02/0302 genotype predisposes its carriers to the early appearance of ICA. There are indications that the disease process is faster and more aggressive in younger children than in older children (Karjalainen et al. 1989), and the risk of developing clinical diabetes is higher for young ICA-positive siblings than for older ones (Thivolet et al. 1991).

Although ICA are sensitive, they showed less fluctuation and transient positivity than IAA in our hands, i.e. they are fairly stable. Because T1D is an incurable disease which requires daily care and entails substantial financial costs, it is essential to identify early as many susceptible children as possible who could benefit from early intervention measures aimed at preventing or delaying progression to the clinical disease. The stability of the screening tool should also be considered, however, since the families in the DIPP study are constantly being informed about the antibody status of their children. Transiently positive and fluctuating antibodies are problematic, as they may elicit unnecessary anxiety within the family.

6.6 Autoantibodies and clinical type 1 diabetes

Progression to clinical T1D was also under observation here. The number of progressors was limited, but some estimates of the relationship of clinical disease and signs of autoimmunity can be made provided that we bear the relatively low number of progressors in mind.

Among the children in whom all four antibodies had been monitored from birth, 1.3% had developed T1D by the age of 5 years, all of these having developed at least two autoantibodies before the manifestation of clinical T1D. The cumulative proportion of children who developed T1D was 7 times higher among the high-risk children than among those with moderate HLA DQB1 risk genotypes. At the beginning of the DIPP study we estimated that approximately 7% of the high-risk children and 2-3% of the moderate-risk children would progress to T1D by the age of 15 years. Based on the present analysis, 7.1% of the former and 2.8% of the latter had developed persistent positivity for multiple ($\geq$2) antibodies by the age of 5 years. We can thus hypothesize that an overwhelming majority of these children with multiple autoantibodies by the age of 5 years will most probably present with T1D by the age of 15 years, given that seroconversions to autoantibody positivity are rare in schoolchildren. The latter assumption is justified, since, as mentioned above, we observed no significant differences in the frequencies of the various antibodies between the DIPP sibs under the age of 5 years and those aged 5 years or older, implying that the majority of susceptible children seroconvert to autoantibody positivity before the age of 5 years.

Both ICA and IAA had a sensitivity of 100% for clinical T1D, whereas the sensitivities of GADA and IA-2A were 85% and 69%, respectively. The specificity ranged from 93% for IAA to 98% for IA-2A, and the positive predictive values for individual antibody reactivities from 16% for IAA to 27% for IA-2A. These predictive characteristics suggest that ICA are the best tool for the primary screening of beta-cell autoimmunity in the DIPP setting. ICA identified all the children who developed multiple autoantibodies by

the age of 5 years, including those who progressed to clinical T1D, and were less frequently fluctuating or transiently positive than IAA. Even though IA-2A had the highest specificity and PPV, it cannot be used as a screening tool alone due to its dubious sensitivity.

7 Summary and conclusions

The present research was conducted within the framework of the Finnish DIPP study, which is globally the most extensive general population-based birth-cohort study focusing on the natural history of T1D. The current work set out to evaluate the role of HLA DQB1 genotypes in the appearance of T1D-associated autoantibodies and the behavior of these autoantibodies in the subclinical phase of the disease and in clinical T1D. ICA were used as a primary screening tool for beta-cell autoimmunity during the immunological follow-up, and their suitability for screening was also evaluated.

The present observations show that the HLA DQB1 genotypes constitute a powerful determinant of beta-cell autoimmunity in non-diabetic children derived from the general population. These genotypes had an effect on both the quantity and quality of beta-cell autoimmunity, implying that the genotyping criteria used in the DIPP study for the classification of HLA-conferred disease susceptibility work accurately, as the signs of autoimmunity increase along with the risk deduced from these genotypes. HLA DQB1 *0602, the dominant protective allele with regard to clinical T1D, appears to be highly protective against destructive beta-cell autoimmunity, since none of the DIPP sibs with this allele had multiple autoantibodies and only occasional ones had single autoantibodies, which could be interpreted as a sign of harmless, transient autoimmunity.

It is essential to start the immunological follow-up early in life in genetically susceptible children in order to try to intervene in the disease process early enough, when there is still sufficient beta-cell mass to preserve some production of insulin. We do not have any clinically applicable measures to stop the development of the disease at the moment, but clinical trials are in process. As shown here, it may be possible to identify the majority of risk children before school age, since the autoantibody frequency rose steadily up to the age of 5 years and the increase appeared to subside after that. Based on the present results it may be estimated that most of the children who develop multiple autoantibodies under the age of 5 years will develop T1D by the age of 15. If preventive measures could be successfully applied to the former group, it would most likely have a conspicuous effect on the incidence of T1D at least in childhood.

We observed that when multiple antibodies appeared they were more permanent, while single autoantibody positivity more easily fluctuated and even disappeared. This pattern supports the earlier notion that persistent positivity for two or more autoantibodies reflects destructive progressive beta-cell autoimmunity, whereas positivity for a single autoantibody represents harmless non-progressive or even regressive beta-cell autoimmunity.

An obvious familial aggregation of beta-cell autoimmunity was seen, although the autoantibodies did not appear at the same time or at the same age in siblings. This difference in the timing of the initiation of beta-cell autoimmunity is curious, since living conditions and dietary factors etc. are largely similar for siblings within the same family. The current observations nevertheless suggest that a longer follow-up might make it possible to define what factors distinguish familial progressors with signs of beta-cell autoimmunity from autoantibody-positive familial non-progressors.

The geographical differences observed here in the distribution of HLA DQB1 risk genotypes and signs of beta-cell autoimmunity offer a fruitful basis for further analysis of this population. As these regional differences could be explained by environmental factors, it is important to analyze the environmental variations between the regions that could lead to such differences in the frequency of humoral beta-cell autoimmunity. It will also be possible in time to determine whether the differences in the appearance of autoantibodies result in similar differences in the development of the clinical disease.

ICA appeared to function well as a primary screening tool for immunological follow-up in our population, having good sensitivity but greater stability than IAA, which had a similar sensitivity. Although ICA also identify children who are likely to have only non-destructive beta-cell autoimmunity, they represent the most common autoantibody specificity in children with multiple autoantibodies and served to identify all the children who developed multiple autoantibodies and all those who progressed to the clinical disease. ICA work well with young children who, as mentioned above, represent a crucial population in attempts to prevent the development of T1D. IAA could be a preferable screening tool for beta-cell autoimmunity, as they are often the first or among the first autoantibodies to appear, but as the parents in the DIPP study are being constantly informed about the autoantibody status of their children, fluctuating and transiently positive IAA could induce unnecessary worry and anxiety.

We also determined the predictive characteristics of the autoantibodies in relation to the development of overt T1D, although the resulting observations have to be considered merely preliminary in view of the limited number of children who developed the clinical disease. IA-2A were the most specific single autoantibodies and had also the highest PPV, but their sensitivity was relatively low, and for that reason they do not represent a suitable primary screening tool. The predictive characteristics obtained suggest that ICA constitute the best tool for the primary screening of beta-cell autoimmunity in the DIPP setting.

In conclusion, the HLA DQB1 genotypes have an impact on both the quantity and quality of humoral beta-cell autoimmunity. The frequency of autoantibodies rises up to the age of 5 years, after which the trend may level off. When multiple autoantibodies appear, they seem to persist and can be regarded as a sign of a destructive disease process. Beta-cell autoimmunity shows clear familial clustering, but the timing of the induction of autoantibody-positivity differs between siblings. Environmental factors are likely to have an effect on humoral beta-cell autoimmunity, since there are geographical variations in the appearance of autoantibodies which cannot explained by differences in the frequency of HLA DQB1 genotypes or in the proportion of affected first-degree relatives. IAA are a sensitive but unstable sign of beta-cell autoimmunity, while ICA are also highly sensitive for the identification of children at risk of T1D and are sufficiently stable. ICA therefore proved to be the best single autoantibody reactivity for use as a method of screening for humoral beta-cell autoimmunity in the present instance.

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Original communications