Preventing the Predictable. Type 1 diabetes in children: Risk factors and impact of participation in prospective follow-up

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FACULTY OF MEDICINE | LUND UNIVERSITY

Preventing the Predictable

Type 1 diabetes in children: Risk factors and impact of participation in prospective follow-up

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LUND
UNIVERSITY

DOCTORAL DISSERTATION
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Title and subtitle: Preventing the Predictable: Type 1 diabetes in children: Risk factors and impact of participation in prospective follow-up

Abstract

Aim: The aim of this thesis was to identify risk factors for type 1 diabetes in children, evaluate the effects of participation in prospective follow-up and the safety of immune tolerance treatment with GAD-Alum in children with islet autoimmunity.

Methods: Subjects from the Swedish DiPiS cohort were studied regarding risk factors, cord blood islet autoantibodies and the effects of early severe life events. We further evaluated morbidity at diagnosis and glycemic control during the first two years of diabetes, comparing the DiPiS follow-up cohort to non-enrolled children. The TEDDY cohort was used to analyze prevalence and effect of analgesic antipyretics before 2.5 years of age on islet autoimmunity at 6 years of age and patterns of analgesic antipyretic use at the study sites. The safety and efficacy of immune tolerance treatment with GAD-Alum were analyzed in a five-year follow-up of at-risk children.

Results: IA-2A autoantibodies in cord blood predicted an increased hazard for type 1 diabetes (HR 6.88; 95% CI 1.46, 32.4; p=0.003.). Parental severe life events after pregnancy predicted type 1 diabetes risk for both the total cohort (HR 1.66; 955 CI 1.02, 2.70; p=0.043) and the DQ2/8 cohort (HR 2.21; 95%CI 1.08, 4.51; p=0.03). The use of analgesic antipyretics did not predict islet autoimmunity at age six years (HR 1.02; 95% CI 0.99, 1.09; p=0.27) but weakly predicted islet autoimmunity at age 3 years. The use of analgesic antipyretics differed between study sites, with a higher prevalence in the US (95.7%) and Sweden (94.8%) than in Finland (78.1%) and Germany (80.2%). Use in the absence of fever was common, especially in the US in comparison to the other sites. Subjects enrolled in DiPiS follow-up were diagnosed with diabetes with less morbidity (p=0.014) and lower prevalence of ketoacidosis (p=0.005). Among subjects enrolled in DiPiS follow-up, HbA1c at diagnosis was lower (9 mmol/mol; p=0.006) and glycemic control was better after diagnosis (12 months, 4 mmol/mol; p=0.009, 24 months, 9 mmol/mol; p <0.001). Immune tolerance treatment with GAD-Alum is safe but does not affect the time to diabetes diagnosis in this cohort.

Conclusion: Early severe life events and IA-2A cord blood antibodies but not early use of analgesic antipyretics may increase type 1 diabetes risk. Severe life events and cord blood autoantibodies are both rare events, and care must be taken in interpreting these results. Participants in prospective follow-up are diagnosed at an early stage, with low morbidity and improved glycemic control, which may be an important factor for recruitment and ethical approval. Immune tolerance with GAD-Alum is safe, but larger, stratified studies are needed to ascertain the possible effects.

Key words: Type 1 diabetes; Risk factors; Prospective follow-up; Immune tolerance; Severe life events

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Preventing the Predictable

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"The prophylactic and etiological treatment of diabetes will surely play an important role in the future, and it is already plain that progress will be along two lines: 1) toward the early detection of the disease, and 2) toward the prevention of the development of the disease in those susceptible to it."

Elliott Joslin, 1916

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Paper	Aim	Methods	Results	Conclusion
I	To analyze possible correlations between the presence of cord blood autoantibodies against GAD65, IA-2 and Insulin and the risk of T1D in the DiPiS cohort.	A Cox proportional hazards model was used to calculate HR for the development of T1D and positivity to cord blood autoantibodies, adjusting for gender, parental T1D, HLA and maternal age.	151 out of 35,683 children had developed T1D by the end of 2013. The presence of IA-2A in cord blood was associated with a seven-fold risk of developing T1D. No increased risk could be seen for GADA or IAA in cord blood.	The study indicates that the presence of cord blood autoantibodies to IA-2 but not GADA or IAA increases the risk of developing T1D.
II	To examine the effects of parenting stress, parental stress and severe life events on T1D risk.	Questionnaire data regarding pregnancy and up to 2 years of life in a cohort of 23,187 children were used in the analysis. Indices for parental discord and parenting stress were created, and scores above the 90th percentile were considered positive. A Cox proportional hazards model was used to calculate Hazard ratios for both the total cohort and a high-risk HLADQ2/DQ8 sub-cohort.	Having experienced a severe life event during pregnancy or during the child's first two years predicted an increased hazard of T1D in both the total cohort (HR 1.7; p=0.043) and the DQ2/DQ8 cohort (HR 2.2; p=0.03). Neither the parental discord index nor the parenting stress index correlated with T1D risk (p=0.53; p=0.90)	The occurrence of a severe life event for the family during the child´s first two years of life correlates with an increased risk of T1D. Parenting stress and parental discord, as reported by the study parents, do not increase the T1D risk of the child.
III	To examine the possible effect on the risk of islet autoimmunity and to describe use and how the use of analgesic antipyretics differs between TEDDY study sites.	Data were collected for 8,542 children in the first 2.5 years of life. Incidence was analyzed using logistic regression, with country and first-child status as independent variables. The time to autoantibody seroconversion was analyzed using a Cox proportional hazards model. For each categorization, a generalized estimating equation (GEE) approach was used.	A higher prevalence of use was found in the US (96%) and Sweden (95%) compared to Finland (78%) and Germany (80%). First-born children were more commonly given acetaminophen but less commonly given NSAIDs. Analgesic antipyretic use in the absence of fever and infection was more prevalent in the US than the other sites. No impact of analgesic antipyretic use could be seen for T1D risk before age six years.	Analgesic use before age 2.5 years is not a risk factor for islet autoimmunity at age six years. The use of ANAP in young children is common and higher in the US than other sites. Use is common even in the absence of fever or infection.
IV	To examine differences in morbidity at diagnosis and glycemic control after diagnosis for participants and non-participants in the DiPiS study.	A comparison of children followed in DiPiS and children without follow-up and risk information. Chi-2 and the Mann Whitney U-test were used to analyze differences between groups.	DiPiS children had lower HbA1c at diagnosis and during regular follow-up at 12 and 24 months. After diagnosis, DiPiS children had fewer symptoms and lower rates of ketoacidosis at diagnosis.	Participation in prospective follow-up before the diagnosis of T1D leads to earlier diagnosis, with fewer symptoms, a decreased incidence of ketoacidosis and better metabolic control up to 2 years after diagnosis.
V	To examine the efficacy and safety of immune tolerance with GAD-Alum in a cohort of children at high risk of T1D with multiple islet autoantibodies but no T1D.	In a double-blind, placebo-controlled clinical trial, children with autoantibodies to GADA and at least one other islet autoantigen received two injections of GAD-Alum or placebo. Children were followed for five years or until T1D diagnosis. Life tables were compared using log-rank $\chi 2$ statistic. The relative risk of diabetes between groups was analyzed using Cox proportional hazards analysis. Mixed-model repeated measures methods were used for secondary outcomes.	A total of 50 children were assigned 1:1 to GAD-Alum or placebo. GAD-Alum did not affect any safety parameter, and no severe adverse events were recorded. Time to diagnosis was not affected by treatment, and treatment did not affect any of the secondary outcome variables.	GAD-Alum, given as two separate doses in a prime and boost fashion in pre-symptomatic children, is safe but does not affect progression to type 1 diabetes.

Abbreviations

ADA	American Diabetes Association
ANAP	Analgesic antipyretic
APAP	Acetaminophen
AUC	Area under the curve
DAISY	Diabetes and Autoimmunity Study
DBS	Dried blood spot
DiAPREV-IT	Diabetes prevention immune tolerance
DiPiS	Diabetes Prediction in Skåne
DIPP	Diabetes Prediction and Prevention
DKA	Diabetic Ketoacidosis
DPT-1	Diabetes Prevention Trial of Type 1 diabetes
FPIR	First phase insulin release
GAD-Alum	Alum-formulated GAD65
GADA	Glutamic acid decarboxylase 65 autoantibodies
HbA1c	Glycated hemoglobin A1c
HLA	Human leukocyte antigen
IA	Islet autoimmunity
IAA	Insulin Autoantibodies
IA-2A	Insulinoma-associated protein 2 autoantibodies
IDAA1c	Insulin dose-adjusted HbA1c
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IvGTT	Intravenous glucose tolerance test
NSAID	Non-steroidal anti-inflammatory drug
OGTT	Oral glucose tolerance test
PCR	Polymerase chain reaction
T_{eff}	Effector T-cell
T_{reg}	Regulatory T-cell
T1D	Type 1 diabetes
TDD	Total daily dose
TEDDY	The Environmental Determinants of Diabetes in the Young
ZnT8RA	Arginine 325 zinc transporter 8 autoantibody
ZnT8WA	Tryptophan 325 zinc transporter 8 autoantibody
ZnT8QA	Glutamine 325 zinc transporter 8 autoantibody

Abstract

Aim: The aim of this thesis was to identify risk factors for type 1 diabetes in children, evaluate the effects of participation in prospective follow-up and the safety of immune tolerance treatment with GAD-Alum in children with islet autoimmunity.
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List of publications

I. **Lundgren M**, Lynch K, Larsson C, Elding Larsson H (2015) Cord blood insulinoma-associated protein 2 autoantibodies are associated with increased risk of type 1 diabetes in the population-based Diabetes Prediction in Skåne study. Diabetologia 58:75–78.

II. **Lundgren M**, Ellström K, Elding Larsson H. Influence of early life stress and severe life events on development of type 1 diabetes in at risk children: The DiPiS study. Submitted for publication. 2017.

III. **Lundgren M**, Steed LJ, Tamura R, Jonsdottir B, Geusaldo P, Crouch C, Sjöberg M, Hansson G, Hagopian W, Ziegler A, Rewers M, Lernmark Å, Toppari J, She J-X, Akolkar B, Krischer J, Haller MJ, Elding Larsson H for the TEDDY study group (2016) High use of Analgesic antipyretics in children. No association to islet autoimmunity: TEDDY study. BMC Pediatrics 2017 May 16;17(1):127.

IV. **Lundgren M**, Sahlin Å, Svensson C, Carlsson C, Cedervall E, Jönsson B, Jönsson, I, Larsson K, Lernmark Å, Neiderud J, Vigård T, Elding Larsson H (2014) Reduced morbidity at diagnosis and improved glycemic control in children previously enrolled in DiPiS follow-up. Pediatric Diabetes 15:494–501.

V. Elding Larsson H**, Lundgren M**, Jonsdottir B, Cuthbertson D, Krischer J. Safety and efficacy of autoantigen-specific therapy with two doses of GAD-alum in children with multiple islet autoantibodies and risk for type 1 diabetes: A randomized clinical trial. Submitted for publication. 2017.

 The papers are referred to by their Roman numerals in the thesis.

Introduction

"Diabetes is a dreadful affliction, not very frequent among men, being a melting down of the flesh and limbs into urine. the patients never stop making water and the flow is incessant, like the opening of aqueducts. Life is short, unpleasant and painful, thirst unquenchable, drinking excessive and disproportionate to the large quantity of urine, for yet more urine is passed. If for a while they abstain from drinking, their mouths become parched and their bodies dry; the viscera seem scorched up, the patients are affected by nausea, restlessness and a burning thirst, and within a short time, they expire."

Aretaeus of Cappadocia (2nd century CE). Adapted from Papaspyros (1952) The history of Diabetes Mellitus.

The history of T1D

Descriptions of diabetes mellitus, or polyuric disease, have existed for at least 3,500 years, as the disease was mentioned in ancient Egyptian papyrus rolls. The name diabetes is derived from the Greek word for syphon or "where the water flows out." Early in the first millennium, it was recognized that polyuric urine tasted sweet, like honey. The "mellitus," or honeyed, adjective was not added to the disease description until the late 1800s. The sweet taste of the urine was identified as glucose in the early 1800s, and shortly thereafter, glucose was shown to be normally present in the blood. In 1880, two distinctive forms of diabetes were proposed by Lancereaux: diabète maigre (lean patients) and diabète gras (obese patients). Early in the insulin age, it was observed that some patients were more or less responsive to insulin. The more insulin-sensitive patients tended to be the lean patients that needed insulin to avoid going into diabetic ketoacidosis (DKA), whereas older, obese patients were insulin-insensitive and ketosis-resistant[1].

Before the 1920s, doctors could do little to counteract the symptoms of diabetes. The clinical course of diabetes patients was short, miserable and inevitably lethal. A multitude of therapies were tested, with exclusion or calorie-restricted diets being the only approaches that had any effect in terms of prolonging the time until the patients died from starvation or DKA. The cause of diabetes still eluded physicians in the early 19th century. Oskar Minkowski (1858-1913) and Josef von

Mering (1849-1908) made a significant breakthrough in the understanding of diabetes in 1889 when they reported that pancreatectomy resulted in severe hyperglycemia in dogs[2].

The concept that internal secretions could direct bodily functions had been proposed at the time, and shortly thereafter, Murray reported that myxedema could be treated with a thyroid extract from sheep. This finding instilled hope that diabetes could also be treated. This goal proved much more elusive, however, as early attempts at insulin treatment all failed. Discouraged, the medical community turned again to diet as a treatment for the disease. The best-known regimen was the starvation regimen of Frederick Madison Allen. This regimen proposed intensive exercise and "as little to eat as possible" and was promoted by Elliott P. Joslin, who was one of the greatest diabetologists of the 20th century. The treatment could in some ways be described as pyrrhic, since the patients could survive for months at the cost of a very low quality of life and died of starvation rather than diabetes.

Insulin was finally discovered by Banting, Best, Macleod and Collip at the University of Toronto in 1921, earning Banting and Macleod the Nobel prize in medicine in 1922. Banting officially decided to share his prize with Best and MacLeod with Collip. Insulin derived from bovine pancreas was first used to treat diabetes patients in 1922, resulting in dramatic results for the first patients, with lowered blood sugar and eliminated glycosuria and ketosis[3,4]. By 1923, bovine insulin was widely available for treatment. The crystallized insulin was impure and had to be injected several times each at great pain. Early treatment advocated resting the pancreas by aggressively lowering blood sugar. This approach combined with available methods to test blood sugar was detrimental to many patients, who suffered greatly from hypoglycemia. Hence, during the early decades of insulin treatment, complications were common and came at an early age. Insulin pharmacology has developed since that time, including delayed action preparations, recombinant human insulin and modern insulin analogs developed via recombinant DNA molecular cloning technology.

The importance of glycemic control was poorly understood during the first decades of insulin treatment, and few physicians thought that glycemic control could prevent diabetic complications[5]. The first good measurement of glycemic control was discovered in 1968 when it was published that glycated hemoglobin (HbA1c) was present in the blood of people with diabetes, representing an objective measurement of glycemic control[6]. However, it was not until the late 1970s, with the introduction of test strips for measuring blood glucose, that regular blood glucose control became practically feasible[7]. The final component of modern diabetes treatment came with the replacement of glass and steel injection syringes with insulin injection pens in the early 1980s and later the portable insulin infusion pump[8,9].

The uncertainty regarding the importance of glycemic control that had plagued parts of the 20th century was finally laid to rest when the Diabetes Control and Complications trial was published in 1993. The study firmly established the current dogma that good glycemic control prevents and delays the progression of microvascular complications in patients with type 1 diabetes (T1D)[10]. Numerous other studies have since confirmed this finding, in both type 1 and type 2 diabetes[11-13].

The current state of diabetes treatment is characterized by slow, step-wise improvements in monitoring, insulin treatment and management.

With the use of insulin Degludec, a long duration insulin, more flexibility and a more consistent insulin profile can be achieved[14]. On the other end of the spectrum, the faster acting insulin Aspart (Fi-Asp) may improve post-prandial glucose control and variability[15]. The evolution of blood glucose monitoring, with continuous and flash glucose monitoring, gives diabetes patients more and faster information about their blood glucose than ever before[16]. Smarter, smaller, more advanced insulin pumps combined with carbohydrate counting would further improve the glycemic control of diabetes patients today.

However, none of these innovations will address the main problem of the diabetes patient: insulin dependency. Although better insulins and technical aids have been developed, a significant portion of patients fail to reach the treatment goals needed to minimize long-term complications[17,18], are forced to work hard to accommodate school days[19] and suffer from higher levels of depression and anxiety than their peers[20]. Unfortunately, even children who manage to reach their treatment goals have double the risk of death from cardiovascular complications[21].

These outcomes highlight the need for continued efforts, not only toward C-peptide conservation for those already affected but also toward exposing the triggering events that start the diabetic immune reaction and preventing the disease process from starting at all.

Diagnosis of Diabetes

Definition of diabetes

The term diabetes mellitus describes a group of metabolic disorders of insulin secretion, insulin action or both. Chronic hyperglycemia is the common denominator among these disorders, although the underlying etiology is diverse. Criteria for the diagnosis of diabetes mellitus, regardless of etiology, have been adopted by the leading world diabetes organizations, as well as the World Health

Organization[22-24]. The diagnostic criteria for diabetes mellitus are summarized in Table 1.

Table 1. Criteria for the diagnosis of diabetes[22,25]

HbA1c ≥6.5% (48 mmol/mol) † **The test should be performed in a laboratory using a method that is NGSP-certified and standardized to the DCCT assay***
OR
Fasting plasma glucose ≥126 mg/dL (7.0 mmol/L)* **Fasting is defined as no caloric intake for at least 8 h**
OR
Two-hour plasma glucose during oral glucose tolerance testing (OGTT) ≥200 mg/dL (11.1 mmol/L) **. The test should be performed using a glucose load containing the equivalent of 75 g of anhydrous glucose dissolved in water or 1.75g/kg of body weight to a maximum of 75 g***
OR
. Classic symptoms of diabetes or hyperglycemic crisis, with **. plasma glucose concentration ≥200 mg/dL (11.1 mmol/L)**

* In the absence of unequivocal hyperglycemia, the diagnosis of diabetes based on these criteria should be confirmed by repeat testing.

† A value of less than 6.5% does not exclude diabetes diagnosed using glucose tests. The role of HbA1c alone in diagnosing T1D in children is unclear and not part of the ISPAD criteria.

The broad classification of diabetes into T1D, with an absolute, eventual, deficiency of insulin secretion, and type 2 diabetes, with a combination of insulin resistance and inadequate insulin secretion, has existed since the mid-20th century. With improved genetic and metabolic testing, the number of different diabetes mellitus diagnoses is increasing. Increased knowledge about the genetic etiology of these disorders has improved our understanding of maturity-onset diabetes of the young (MODY), neonatal diabetes, and diabetes caused by defects in insulin action[26-29] (Table 2.).

To improve the diagnosis of T1D, additional diagnostic tools besides plasma glucose can be used:

- Islet autoantibodies: 96-98% of newly diagnosed children with T1D are positive for at least one islet autoantibody (IAA, GADA, IA-2A or ZnT8A), all but confirming the diagnosis[30-34].
- C-peptide: Measuring fasting C-peptide can indicate whether or not the patient is insulin-deficient. However, measurements during the initial period after the T1D diagnosis can be hard to interpret due to remission of insulin secretion. This test can still be an efficient tool to discriminate between T1D, type 2 diabetes and monogenic diabetes[35,36].
- HLA genotype: In those patients in which the HLA genotype is analyzed at the time of diagnosis, a very low- (HLA DQ6.2) or low-risk genotype warrants further testing if combined with negative islet autoantibodies[37,38].

In children without islet autoantibodies, a young age (<6 months), autosomal dominant diabetes in the family, associated conditions (blindness, deafness, and syndromic features) or exposure to drugs related to insulin resistance, further testing should be considered[39-41].

Table 2. Classification of diabetes (adapted from the American Diabetes Association and ISPAD[22,25])

Type 1 diabetes		
Type 2 diabetes		
Other specific types of diabetes	**Genetic defects**	**MODY 1 (chromosome 20, HNF-4α), MODY 2 (chromosome 2, glucokinase), MODY 3 (chromosome 12, HNF-1α), MODY 4 (chromosome 13, IPF 1), MODY 6 (Chromosome 2, NeuroD1), MODY 7 (chromosome 9, CEL)**
		Transient neonatal diabetes (Imprinting defect on 6q24; mutations in ABCC8 or KIR6.2)
		Permanent neonatal diabetes (WRS, mutation in EIF2AK3; IPEX syndrome; mutations in INS and FOXP3)
		Mitochondrial DNA mutation
		Others
	Genetic defects in insulin action	**Type A Insulin Resistance, Rabson Mendenhall Syndrome, Leprechaunism (mutations in INSR)**
	Diseases of the exocrine pancreas	**Pancreatitis, Pancreatectomy, Cystic fibrosis, Hemochromatosis and others**
	Endocrinopathies	**Acromegaly, Cushing´s syndrome, Glucagonoma, Pheochromocytoma, Hyperthyroidism, Hyperthyroidism, and others**
	Drug or chemical induced	**Glucocorticoids, Thyroid hormone, Diazoxide, B-adrenergic Agonists, Thiazides, Phenytoin, Γ Interferon, Nicotinic acid, Vacor, and others,**
	Infections	**Congenital Rubella, CMV, others**
	Uncommon forms of immune-mediated diabetes	**Stiff-man syndrome, Anti-insulin receptor antibodies, Autoimmune Polyendocrine Syndrome (APS) Types I and II, and othersand others**
	Other genetic syndromes	**Down syndrome, Klinefelter syndrome, Turner syndrome, Wolfram syndrome, Friedrich ataxia, Huntington syndrome, Prader Willi syndrome, and others**
Gestational Diabetes		

CEL, carboxyl ester lipase; HNF, hepatocyte nuclear factor; IPEX, immunodysregulation polyendocrinopathy enteropathy X-linked syndrome; IPF, insulin promoter factor; MODY, maturity-onset diabetes of the young; *PAX4*, Paired Domain gene 4.

Stages of T1D

It has long been known that a long prodromal period precedes the clinical diagnosis of T1D. Initially, this period was thought to be a matter of months, during which incidental dysglycemia occurred due to decreasing β-cell reserves.
The current understanding of islet autoimmunity (IA) changes the previous notion of T1D as a chronic disease with acute onset into a slowly progressing, immune mediated β-cell destruction. The etiology is not known but recent data suggest that different triggering events may induce either IAA or GADA as the first appearing autoantibody[42-44][45]. IAA appear first in children with the DR4-DQ8 haplotype while GADA appear first in DR3-DQ2 children. A first autoantibody is often followed over months and years to a second, third or fourth autoantibody independent of the HLA type of the child. The progressive pathogenesis is associated with β-cell loss eventually leading to dysglycemia and hyperglycemia. This change has also lead to a new classification of the early stages of T1D, including the period of IA, into three stages, from IA to overt, clinical disease[46](Figure 1.). However, taking the concept of pre-symptomatic T1D one step further and accepting IA as a disease entity in itself is currently under debate[47,48].

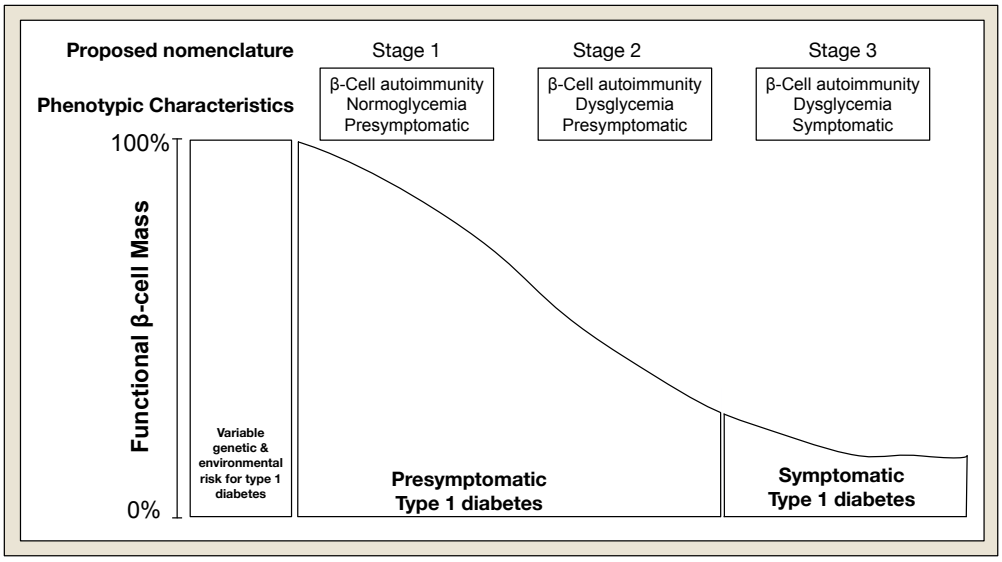

Figure 1. Stages of Type 1 diabetes.
According to the 2015 statement of ADA/JDRF/Endocrine Society. Adapted with permission from Insel RA and colleagues[46].

Stage 1: IA, with persistent seropositivity for at least two islet autoantibodies but with normal glucose and normal HbA1c. The β-cell mass is still large enough to maintain normoglycemia. In individuals with genetic HLA risk, the risk of developing symptomatic T1D in this group is approximately 44% in 5 years and 70% in 10 years. The lifetime risk approaches 100%[49-51].

Stage 2: IA, with persistent seropositivity for at least two islet autoantibodies and glucose intolerance/dysglycemia. Dysglycemia in this stage is most often defined as either impaired glucose tolerance (IGT), with elevated 2-hour plasma glucose values ≥ 7.8 mmol/L, or elevated glucose levels at intermediate time points (plasma glucose ≥ 11.1mmol/L) on a standardized OGTT and/or HbA1c ≥ 39 mmol/mol. The two-year risk of symptomatic disease for this phase is estimated at 60%, and the 4- to 5-year risk is 75%[52].
Stage 3: Symptomatic disease. T1D with classical signs of the disease: polyuria, polydipsia, weight loss, fatigue and possible metabolic decompensation and DKA.

The staging of T1D, which further signifies this condition as a progressive disease for which symptomatic disease is the end point, allows for earlier attempts at prevention and intermediate, more efficient, endpoints in clinical trials[53]. Staging also allows for early diagnosis, significantly decreasing the rate of metabolic decompensation and DKA[54-57]. Whether early diagnosis benefits patients and their families psychologically remains unclear and must be further addressed[58-60].

Epidemiology of T1D

The incidence rate suggests a diabetes epidemic

Childhood diabetes has been recognized for hundreds of years. However, reliable global incidence data for T1D have not been available until the late 20th century. In the 1980s, the diabetes hotspots in Finland and Sardinia were not yet recognized, and incidence estimates were not available for 90% of the world's population[61]. Diabetes incidence reports from the early 1900s are rare and of limited scope. However, using existing data, one could assume a rising incidence. For example, the incidence appears to have risen from 2 to 7 cases per 100,000/year between 1900-1920 in Norway and from 2 to 4 cases per 100,000/year in Denmark[62]. Hence, childhood diabetes appears to have been a rare condition at the start of the 20th century.

More reliable incidence data became available later in the 20th century. The US national health survey during the 1930s reported incidence numbers of 0.35-0.41/1,000 for children younger than 15 years[63], and additional data from Norway, which were collected between 1925 and 1954, support an estimated incidence of 4.1/100,000[64]. In the Norwegian data, it is also interesting to note that no discernible increase in incidence was seen during the 30-year study period. Early Finnish data are hard to interpret and compare to the present-day situation due to the hardships of the 1930s and 1940s. More reliable data from 1953 estimate the Finnish incidence at 12.5/100,000/year, a meager number in comparison to the current incidence[65].

When this increase in incidence levels occurred is not easy to pinpoint. It has been proposed that the incidence increased during the 1950s[66]. The previously mentioned Norwegian study was later extended for another 10 years. At the end of this period, the incidence numbers had climbed to 8.4/100000/year[67]. Data from Danish sources paint a similar picture, with an approximately doubling of the childhood diabetes incidence[68].

Better data are available from the later part of the 20th century. In a comprehensive review of 37 studies performed between 1960 to 1996, a rising trend was noted in 24 studies, with an average estimated annual increase of 3%[62]. This number is also supported by later data[69]. The extrapolation of these studies predicted a 40% increase in the childhood T1D incidence until 2010 and a possible rise of 70% between 2005 and 2020[70] (Figure 2.).

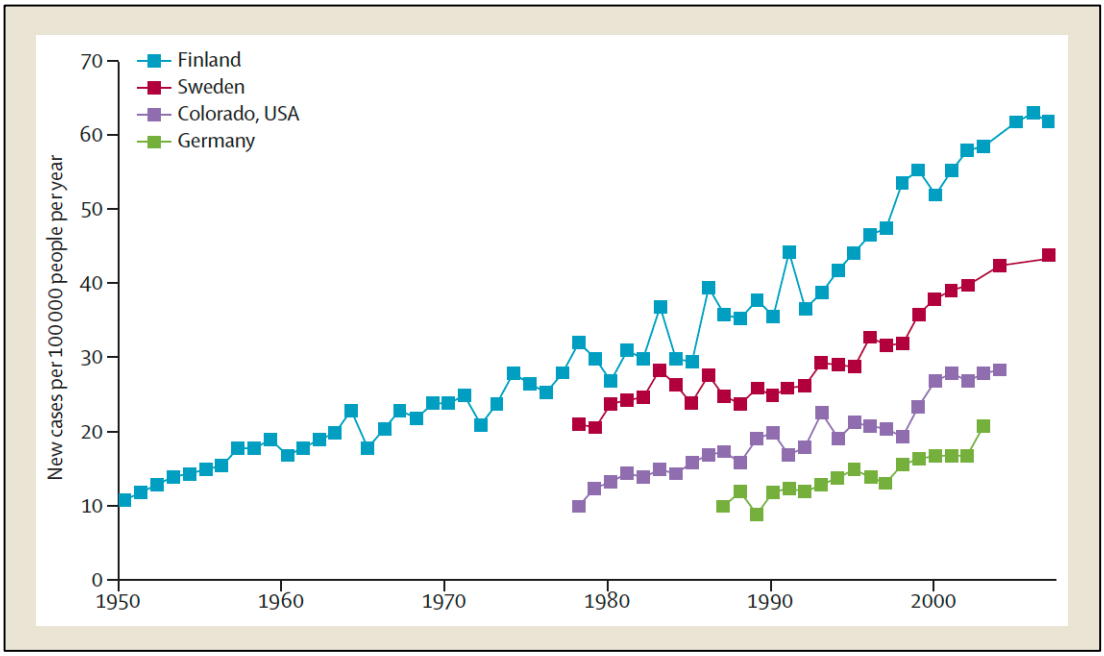

Figure 2. Incidence of T1D in children aged 0-14 years over time. Time-based trends in the incidence of T1D in children ages 0-14 years. Reproduced with permission from Atkinson and colleagues.[71-74]

In the global setting, the T1D incidence varies significantly, with a difference of over 400-fold between countries[75]. Generally, T1D is uncommon in Asia and South America, with incidence rates of approximately 0.1/100,000, and vastly more common in Finland and Sardinia, with an incidence rate of 50/100,000. However, the distribution is more complex, as countries in close proximity have very different incidence numbers.

In-country variations in incidence

The incidence of T1D differs not only between countries but also within countries and tends to increase through childhood, with a peak between 10-15 years[76].

However, at present, there are indications that a shift towards the younger age groups is occurring, with higher incidence rates in the 0- to 4-year group[77 78]. Generally, incidence rates peak at puberty and are lower after age 15[79]. Significant differences also exist within countries, with 1.5-fold differences described in Sweden and Norway, with clusters of higher incidence, as well as a north-south gradient in incidence rates[80,81].

There are small differences in incidence rates between the sexes. Generally, the incidence peak in children occurs earlier in girls, but the general differences in the age group between 0-15 years are small. After puberty, a male predominance of 1.3-2 to 1 is present in many populations[79,82].

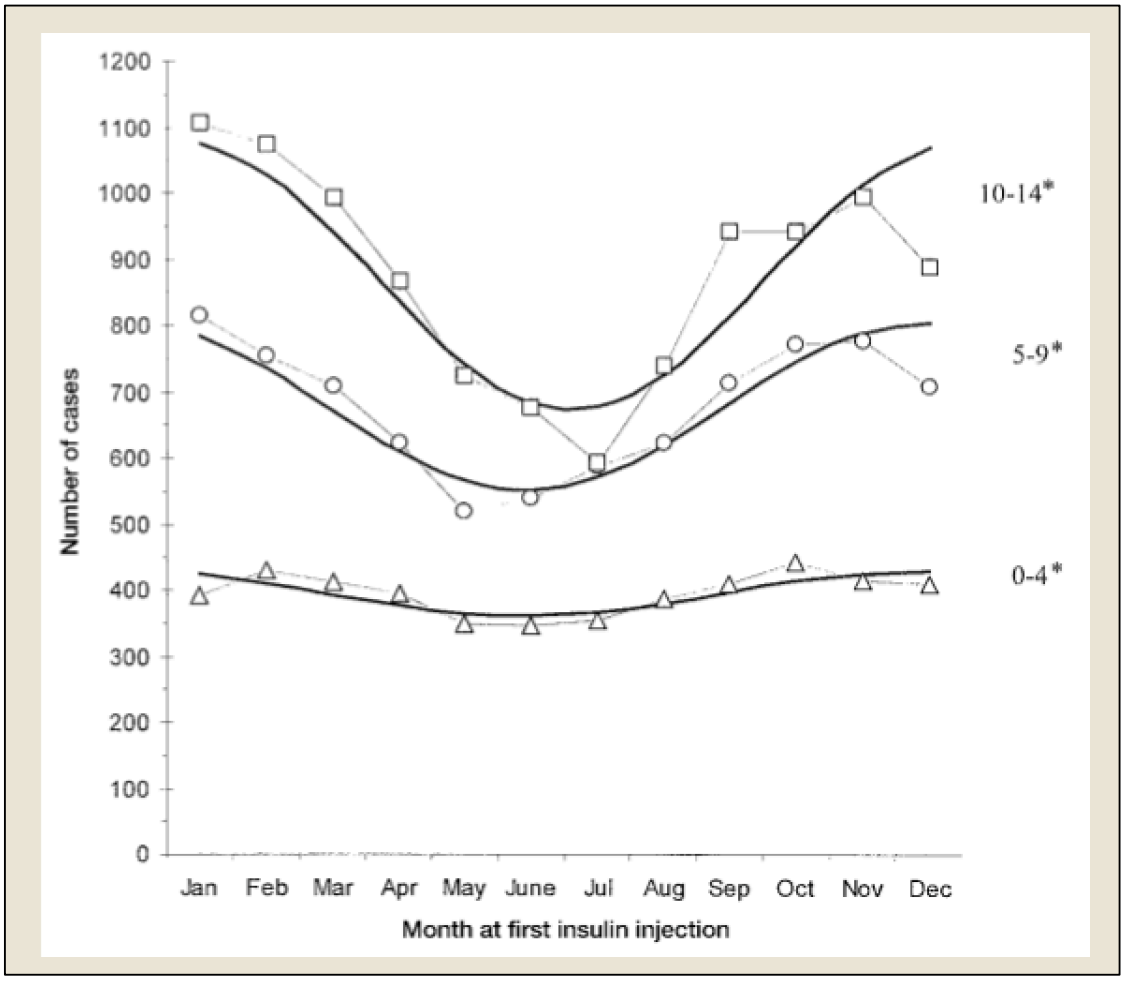

Figure 3. Seasonality at first insulin injection. Stratified by age group.
Reproduced with permission from Green A and colleagues[83].

The incidence of T1D differs by seasonality as has been described by several studies[84]. More patients are diagnosed during the winter and autumn months, a phenomenon that is present with small variations between countries. This seasonal variation also seems to be more significant in the older age group, in which children are between 10 and 14 years at diagnosis (Figure 3).

Pathogenesis of T1D

Many journal articles on T1D start by stating that it is a chronic, autoimmune disease of β-cell destruction. This statement is based on the presence of insulitis, inflammation of the pancreatic islets, first reported over 50 years ago[85]. A model for the development and pathogenesis of T1D was proposed in the mid 1980´s[86].According to this hypothesis, individuals have a fixed number of β-cells at birth. A trigger, possibly a virus, induces cellular damage and as a result β-cell autoimmunity. Activated, auto-reactive, T-cells destroy β-cells leading to a continuously decreasing ability of insulin secretion. When approximately 10% of the β-cells remain, hyperglycemia and T1D diagnosis is reached. That this model does not paint a complete picture of T1D pathogenesis has been proven since its publication, although parts of it is still valid.

The anatomical location of the pancreas has hampered the histological and functional evaluation of the pancreas itself during T1D progression. Until recently, studies of pancreatic pathology have had to rely on specimens from T1D patients obtained at autopsy. The nPOD cooperation, however, focus on collecting pancreatic tissue from deceased patients with T1d as well as subjects with IA[87]. At diagnosis approximately 70% of the pancreatic islets display an absence of insulin but some patients display islets without any signs of inflammation[88]. Islets deficient of insulin, are significantly less likely to display signs of insulitis[89]. The number of lost β-cells at diagnosis also seem to vary, where once thought to amount to about 90%, as little as two thirds of the β-cells lost have been found in newly diagnosed T1D patients. The destruction of β-cells is very selective, leaving the other hormone producing cells of the islets (α-cells, γ-cells and PP-cells) unaffected[90]. The insular infiltrates of the pancreas consist mainly of CD8+ T-cells as well as macrophages, CD4+ T-cells, B-lymphocytes, and plasma cells[90]. This, together with the dominant role of HLA in T1D risk as well as ample experimental and clinical results, is the basis of the concept of a T-cell driven disease[91].

One model of cellular autoimmunity in T1D proposes that after the initial trigger damages β-cells they undergo apoptosis. This makes the intracellular components available to the immune system after being absorbed by antigen presenting cells, among them dendritic cells. These cellular components are then presented to T-cells using the HLA heterodimers on the cell surface of antigen presenting cells. CD4+ T-cells, T-Helper cells, activate both macrophages, CD8+ T-cells as well as B-cells. B-cells start producing antibodies to the presented cellular components leading to islet autoantibodies whereas CD8+ T-cells attack β-cells leading to further damage and insular infiltration[92].

Several alterations to the previously established model of T1D pathogenesis has been proposed but as of yet no new unifying theory has been proposed. Several factors are still undetermined in the pathogenic process, which will need to be

further addressed. The curious specificity of β-cell death, with remaining endocrine cells being unaffected in the islets, is still not explained[93]. Additionally, the triggering event leading to autoimmunity still eludes us. The heterogeneity of β-cell death, and possibly resurrection, is also unknown, where some T1D patients have remaining insulin producing β-cells for many years after T1D diagnosis[88].

Giving a full account of all aspects of T1D is well beyond this thesis. The focus of the latter parts of this chapter will focus on factors related to prediction and prevention, although at the same time tightly connected to our understanding of T1D pathogenesis.

T1D risk assessment

Autoantibodies

Seroconversion

Our knowledge of islet autoantibody seroconversion in children has expanded significantly during recent years. We now know that seroconversion occurs early, often during the first year of life, but can occur significantly later[45,94] (Figure 4.). The most common first islet autoantibody is IAA, and seroconversion to GADA tends to be a later phenomenon[95] (Figure 5.). A single islet autoantibody correlates with a small increase in T1D risk, but we also know that a portion of children revert back to antibody negativity and return to their previous T1D risk level[96]. Further development of islet autoantibodies happens in a sequential manner; the risk of multiple autoantibody positivity is significantly lower if a second islet autoantibody does not appear during the first year after initial seroconversion[97].

Islet cell autoantibodies

The association between T1D and islet autoantibodies has been recognized since the 1970s[98,99]. At that time, islet cell antibodies (ICAs) were identified in newly diagnosed T1D patients. ICAs were later shown to be present earlier in the disease process, an indicator of the long period of IA leading up to clinical diagnosis[100,101]. ICAs were found in approximately 80% of newly diagnosed T1D patients, making them a useful instrument for prediction[98]. However, the method was troublesome in that it required human pancreatic tissue from blood group O donors for analysis[102]. ICA-staining was eventually found to be attributed partly to autoantibodies against IA-2, GAD65 or possibly insulin[103-106]. Given the methodological and sensitivity/specificity-related problems associated with ICA analysis, its use is now limited. However, studies show that up to 5% of newly diagnosed children who are negative for GADA, IAA, IA-2A and ZnT8A test

positive for ICAs, indicating the possible presence of other unidentified autoantigens with predictive properties[107,108].

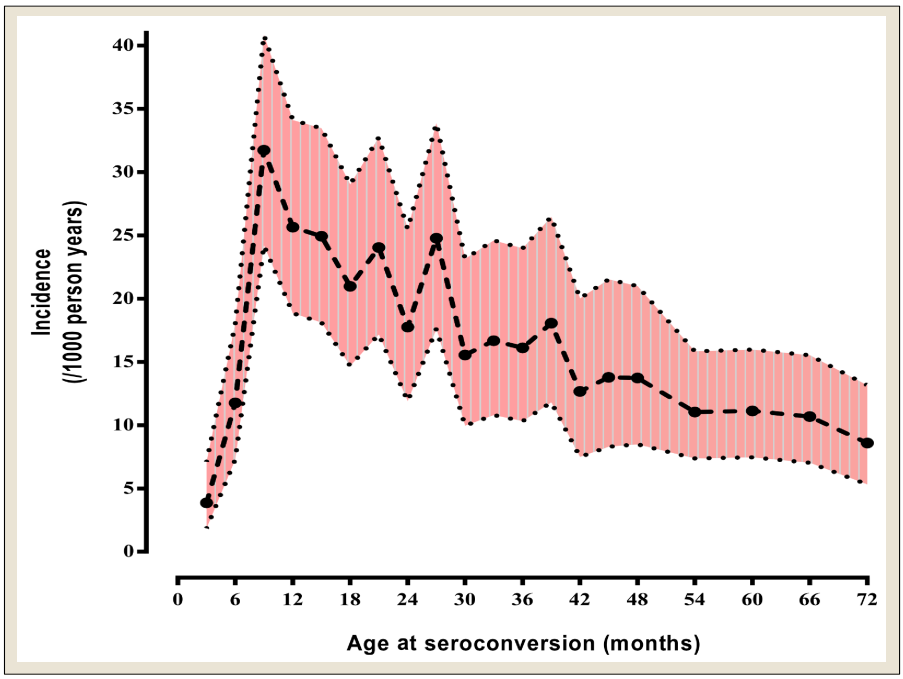

Figure 4. Age at islet autoantibody seroconversion.
Incidence in children aged 0-6 years in the TEDDY study. Printed with permission from Krischer and colleagues[44].

Insulin autoantibodies

Insulin autoantibodies (IAA) specific for insulin and the insulin precursor pro-insulin were first described in 1983[109]. IAA is the earliest autoantibody to appear, in many patients before the age of two years[45,95]. Approximately 90% of children who are diagnosed with diabetes before the age of five years are positive for IAA, compared to less than half of children who are diagnosed after puberty[51]. Hence, a peak in seroconversion to IAA positivity seems to occur prior to one year of age, and after this age, the number of seroconverters declines[45] (Figure 5). A correlation also exists with HLA DR4-DQ8, with a significantly higher incidence of IAA positivity among these children[110]. IAA radioimmunoassays have high sensitivity and specificity for T1D. However, unfortunately, the analysis has poor inter-laboratory concordance, making standardization difficult[111].

GAD autoantibodies

Autoantibodies specific for the 64k protein were first described in 1982 and later shown to be directed at glutamic acid decarboxylase (GAD)[103,112]. GAD is an enzyme that is present in neurons and β-cells and produces the neurotransmitter gamma-aminobutyric acid (GABA). GAD-autoantibodies (GADA) are directed against the 65K isoform of the protein, which is expressed primarily in human β-cells[113]. GADA is present in up to 80% of newly diagnosed children with T1D. The incidence of seroconversion to GADA rises during the first 3 years of life and

then appears to remain relatively constant during early childhood[45] (Figure X). GADA also exhibits a more persistent positivity, even when β-cell function has been severely compromised.

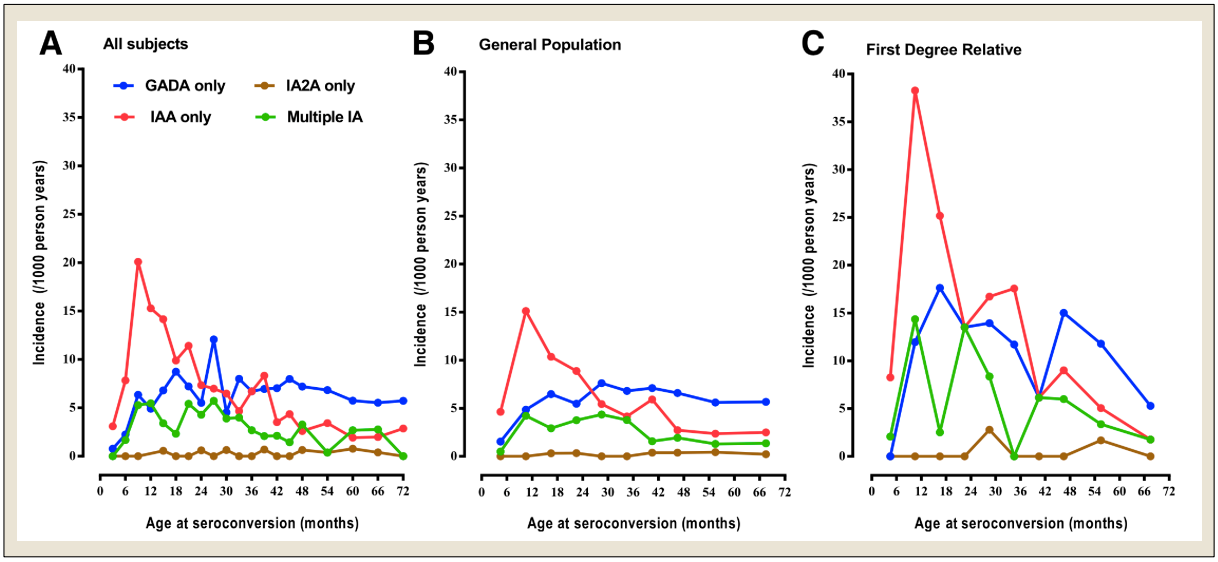

Figure 5. Incidence of IAA only, GADA only, and IA-2A only as first-appearing IA and multiple IA at seroconversion. Results are shown for the total cohort (A) and for the general population (B) and first-degree relatives (C) separately. Reprinted with permission from Krischer and colleagues[44].

This finding, combined with a strong correlation with C-peptide levels, makes GADA a good candidate for prediction efforts[114,115]. GADA is associated with the high-risk haplotype HLA DR3-DQ2[45,95]. More recently, the possibility of further increasing the sensitivity and specificity of GADA has been investigated using N-terminally truncated GADA and anti-idiotypic GADA[116-118]. Standardized GADA assays using radio-binding assays or ELISA have been standardized and show good concordance between laboratories[119].

IA-2 Autoantibodies

The autoantigen for autoantibodies to insulinoma-associated protein 2 (IA-2), which was previously known as ICA512 and first described in 1994, is a tyrosine phosphatase-like enzyme[120-122]. The enzyme is present in two isoforms in pancreatic α- and β-cells, as well as in neuroendocrine tissue. Like the GAD and ZnT8 antibodies, IA-2 antibodies recognize a cytoplasmic, intracellular part of the protein: the C-terminal region[105]. IA-2 is typically not the first appearing autoantibody, and only 9% of children seroconvert to IA-2A as their first islet autoantibody. However, cases in which the initial seroconversion is to IA-2 are associated with a more rapid progression to T1D[45,51,95]. Higher titers of IA-2 have also been attributed to a more rapid progression to clinical diabetes[123-125]. IA-2A positivity has been reported to be inversely correlated with HLA DR4-DQ8 and correlated with the DRB1*0401 allele[126]. In contrast, the DR3-DQ2 haplotype was negatively associated with IA-2A [127]. Standardized radio-binding assays for IA-2A

present good concordance between laboratories, demonstrating high sensitivity and specificity[128,129].

ZnT8 Autoantibodies

The cation efflux transporter zinc transporter 8 (ZnT8) has recently been described as a target autoantigen in patients with T1D[130]. This protein is involved in zinc-insulin crystallization, as well as insulin secretion, by accumulating zinc into the insulin granulae[131]. There are three isoform variants of the ZnT8 autoantibody, which are directed at different variants at position 325 in the protein: ZnT8RA (arginine 325 Zinc transporter 8 autoantibody), ZnT8WA (tryptophan 325 Zinc transporter 8 autoantibody) and ZnT8QA (glutamine 325 Zinc transporter 8 autoantibody). Autoantibodies to any of these isoforms represent additional markers of T1D risk[132,133]. Up to 80% of new-onset pediatric T1D patients are positive for ZnT8A, including 26% of patients who are negative for GADA, IA-2A and IAA[34,107,134-137]. Among newly diagnosed T1D patients from 0.6 to 58 years of age, ZnT8RA was present in 53%, ZnT8WA in 44% and ZnT8QA 34%, respectively[138]. ZnT8A titers tend to increase with age and have a higher prevalence, with a peak in late adolescence[137]. ZnT8A positivity has been reported to predict a more rapid progression to diabetes but can also persist for many years and often remain at diagnosis[34,130,139]. However, after diagnosis, titers of ZnT8A rapidly decline. Approximately 40% of ZnT8A-positive patients become negative 5 years after diagnosis[140,141]. At 25 years post-diagnosis, only 7% of T1D patients remained ZnT8A-positive, compared to 26% for GADA and 20% for IA-2A. Radio-binding assays for ZnT8A were standardized and validated in the international DASP workshop on islet autoantibody concordance[142].

Novel minor autoantigens

In addition to the previously mentioned T1D-related autoantigens, several other minor autoantigens have been described. These autoantigens include candidate autoantigens that may be associated with β-cell autoimmunity and T1D. Some of the present candidate autoantigens include Tetraspanin 7[143,144], Neuropeptide Y[145], HSP60[146], ICA69[147], INS-IGF2[148], Carboxypeptidase H[149], vesicle-associated membrane protein-2 (VAMP2)[150], ICA/SOX13[151], glima-38[152], GLUT-2[153] and imogen 38[154].

HLA

More than 40% of the genetic susceptibility to T1D can be explained by the HLA genes[155,156], representing three classes: Class I, Class II and Class III, all located on the short arm of chromosome six. The HLA genotype affects the configuration of the peptide binding groove of the HLA molecule on the surface of antigen presenting cells, determining which peptide will be bound in the groove and presented to T-cells (Figure 6.). Hence, allelic variations in the HLA coding region affect antigen recognition and presentation to the immune system through the interaction between the HLA molecules and the T-cell receptor of T-cells[157,158]. The HLA class II region has three regions: DR, DQ and DP. The HLA DQ molecule is a heterodimer, consisting of an α-chain encoded by the DQA1/A2 allele and a β-chain encoded by the DQB1/B2 allele[159]. For categorization, each amino acid variant of the α- and β-chains is given a four digit number, preceded by the gene designation (e.g., DQA1*05:01-DQB1*02:01)[160]. The alleles within this particular location are often inherited together as haplotypes, in linkage disequilibrium as the distance between the alleles are very short and that recombination events therefore are reduced. This is of particular importance as e.g. HLA-DQ A1 and B1 allele are especially polymorphic[161].

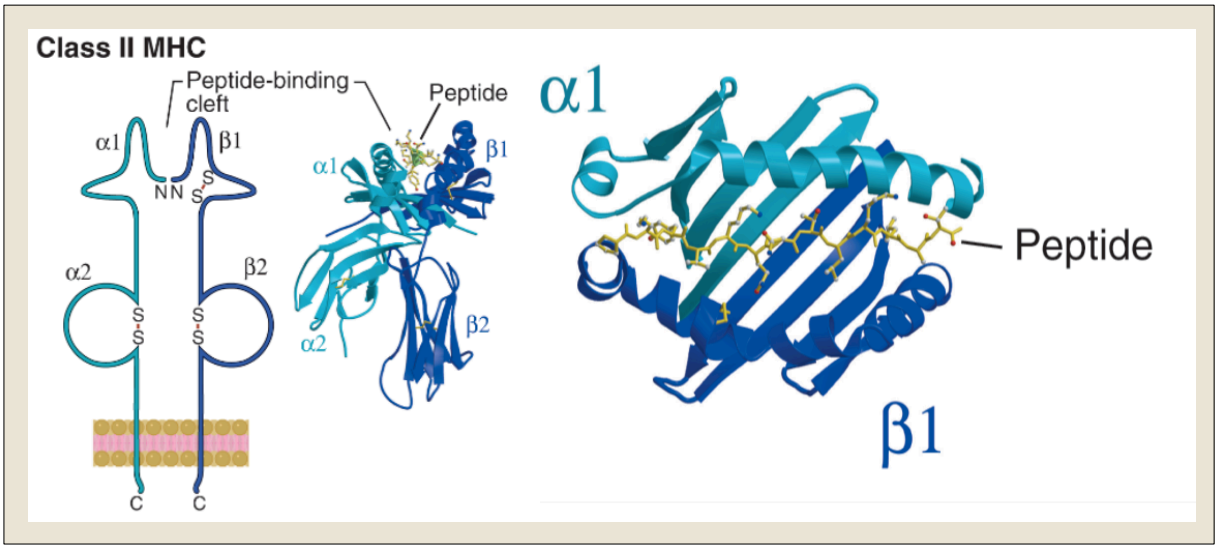

Figure 6. Structure and binding of Class II MHC molecules.
Schematic diagram and crystal structure of the HLA class II molecule with the peptide binding cleft (left). Peptide bound on the floor of the HLA class II peptide binding cleft (right). Reproduced with permission from Abbas and colleagues, Basic immunology 4th ed.[162]

The HLA-DR and HLA-DQ alleles have been shown to be the major determinants of HLA-derived T1D risk and can confer a protective, neutral or susceptible effect. However, the risk conferred by the HLA haplotype is not constant between populations or between ethnic groups[163]. The highest risk haplotypes are conferred by HLA DR3 (DRB1*03*)* together with DQ2 (DQA1*05:01-DQB1*02:01) and by DR4 (DRB1*04) together with DQ8 (DQA1*03:01-DQB1*03:02)[164,165](Table 3.).

The highest risk genotype (HLA DQ2/8) is found in approximately 3% of the Swedish population, and one of the DQ2 or DQ8 haplotypes is found in approximately 50% of the Swedish population. It has previously been shown that one of the HLA haplotypes DQA1*0301, DQA1*0501, DQB1*0302 or DQB1*0201 is present in 97% of new onset diabetes patients in Sweden, and the heterozygous genotype DQ2/8 is present in 24-30% of new-onset diabetes patients in Sweden[166-169]. Hence, only 3% of new diabetes patients carry one of the other, lower-risk haplotypes.

Table 3. Absolute risk for T1D according to HLA DR/DQ genotype. Adapted and reprinted with permission from Haller MJ and colleagues[170].

DQ/DQ	Risk	First-degree relative	General Population
DR 3/4, DQ 0201/0302	High	1/4-5	1/15
DR 4/4, DQ 0302/0302	High	1/6	1/20
DR 3/3, DQ 0201/0201	High	1/10	1/45
DR 4/X, DQ 0302/X (X≠0602)	Moderate	1/15	1/60
X/X	Low	1/125	1/600
DR 0403 or DQ0602	Protective	1/15,000	1/15,000

However, the risk conferred by HLA is not static over time or equal across populations. During recent decades, a significant increase in T1D incidence has occurred, especially among the youngest children[77,83,171]. However, the contribution of HLA to T1D risk is spreading to other genotypes and haplotypes. For example, in 1987 HLA-DQ2/2 were not associated with increased risk but in 2017 this genotype has a significant risk. This finding further emphasizes the important contribution of gene-environment interactions[172,173]. It has been proposed that the HLA genotype directly affects both the progression to diabetes and the age at onset, with a stronger contribution when T1D is diagnosed in childhood[164,174-178]. There also seems to be an ethnically correlated factor related to HLA T1D genetic risk[179]. For example, even though non-Hispanic whites and Hispanics have been shown to have the same frequencies of specific alleles and haplotypes[180-182], the relative haplotype risk differs between the two groups[183].

HLA has been and will continue to be one of the most important factors for early T1D risk estimation. The strong contribution of this region to risk combined with cost effectiveness makes analysis suitable for large-scale screening. However, newer models that include non-HLA genetic markers will surely improve early prediction further in the near future[184-188].

Non-HLA Genetic risk factors

The genetic component of T1D cannot be explained by HLA-derived effects alone. To date, more than 50 additional genetic loci have been associated with

T1D risk in genome-wide association studies (GWAS)[189]. The functions of many of these candidate genes and single nucleotide polymorphisms remain unclear. However, improved assays and methods are adding to our current understanding of gene interactions in the context of T1D risk. Some of the current candidate genes are presented in Figure 7.

INS

One of the earliest non-HLA candidate genes to contribute to T1D risk is located at or near the INS gene on chromosome 11p15.5[190]. Near the insulin gene lies a region with a variable number of tandem repeats (VNTR) whose class I alleles are attributed with an increased risk of T1D. This region has been associated with lower insulin mRNA and protein expression in the thymus in comparison to the dominant, protective class III alleles. This reduced expression decreases central tolerance and increases susceptibility to disease[191,192]. The putative role of insulin as the major initiating autoantigen in T1D also lends support to this hypothesis[193].

PTPN22

The gene for tyrosine phosphatase (PTPN22) on chromosome 1p13 is associated with several autoimmune diseases, including T1D[194,195]. LYP, which is encoded by the PTPN22 gene, inhibits T-cell activation and may be one of the factors responsible for increased T1D risk.

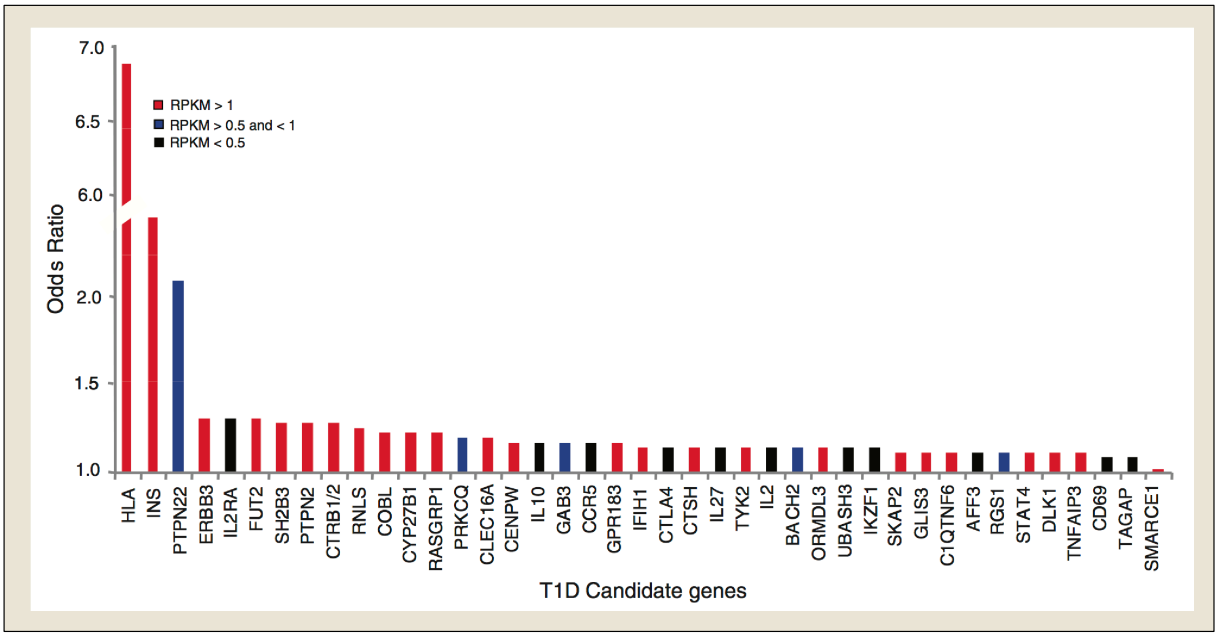

Figure 7. Expression of candidate T1D genes in human pancreatic islets.
Candidate risk genes for T1D, ranked by the odds ratio conferred by the risk allele. Genes expressed in the pancreatic islets (Blue: low expression; Red: medium-high expression) or not expressed in the pancreatic islets (Black) are color-coded in the figure. Reprinted with permission from Santin and colleagues[196].

CTLA-4

Cytotoxic T-lymphocyte-associated protein 4 is a co-stimulatory receptor that inhibits T-cell activation and functions in CD4 T-regulatory cells. Variations in this gene have a strong effect, possibly via the regulation of peripheral tolerance, and this gene is also associated with several other autoimmune diseases[197,198]. HLA and CTLA-4 alleles revealed support for a bidirectional trigger for either IAA or GADA as a first appearing β-cell autoantibody in early life [42].

IL2RA

IL2RA encodes CD25 on naive T-regulatory cells, memory T-cells and monocytes[199]. The regulation of the CD25 protein is important for the suppression of T-cell proliferation by an immunogenic stimulus[200,201], and IL2RA has also been implicated in the pathogenesis of other autoimmune diseases[202].

Environmental risk factors

The increase of T1D that has occurred during recent decades cannot be explained by genetic factors alone. The impact or presence of some environmental factor must have changed. It is also possible that the impact differs based on the HLA-DQ genotype, which may explain some of the dilution of the high-risk HLA genotypes[44]. That environmental factors play a role in the risk of T1D has been postulated for a long time, but the responsible risk factors have proved elusive. However, there is strong support for the impact of environmental factors. In Europe, populations that are genetically close but separated by socioeconomic borders have significantly different T1D incidence rates[203], and we also know that immigrants tend to acquire genetic risk levels similar to those of the inhabitants of their new homeland, despite coming from low incidence areas[204]. Through migration and free movement, diabetes risk is becoming more homogenous, despite the stability of genetic factors[205].

A plethora of environmental factors have been proposed to influence the risk of T1D and will be summarized in this chapter (Figure 8.). A more thorough review of all of these risk factors is beyond the scope of this thesis. For the sake of clarity, a separation between the factors thought to initiate IA and the factors that accelerate and support the progression to clinical diabetes will be made, to the extent that current evidence allows.

Prenatal risk factors

One of the earliest described prenatal risk factors for type one diabetes is congenital rubella[206], with approximately 20% of affected children developing a T1D-like disease. Other factors that have been reported to increase T1D risk include a higher relative birthweight[207,208] and delivery by cesarean section [209],

although both of these factors have weak and conflicting supporting evidence. High maternal age has also been shown to increase T1D risk in several studies[210]. Additionally, maternal enterovirus infection during pregnancy has been implicated as a risk factor for T1D[211].

The association between vitamin D levels in pregnancy and T1D risk has also been studied, with some studies showing a protective effect of higher maternal intake of vitamin D[212,213]. These results are equivocal, with meta studies reporting no protective effect[214].

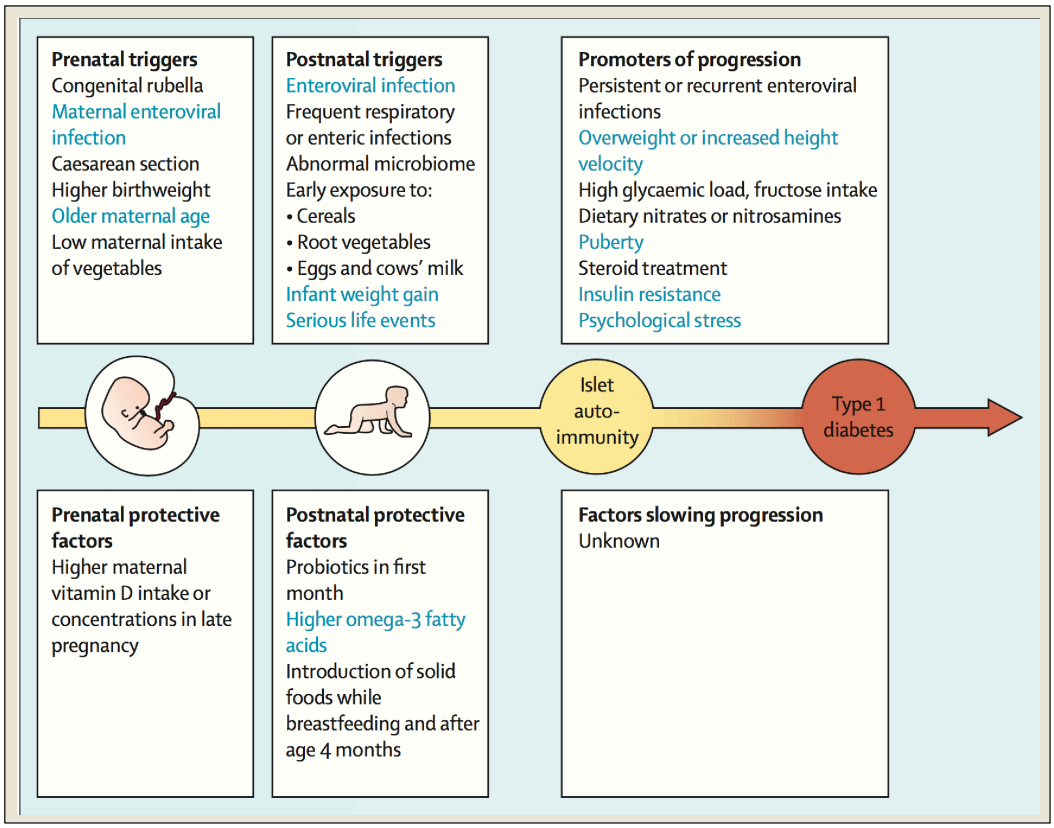

Figure 8. Environmental triggers and protective factors for IA and promoters of progression to T1D.
Reprinted with permission from Rewers and colleagues[215].

Postnatal risk factors

The most researched risk factor for IA and T1D in the postnatal period is viral infections. Several aspects of enteroviral (EV) infection have been studied. Enteroviruses can cause pancreatitis and have been found in the pancreas in T1D patients[216,217]. These viruses also exhibit a tropism for pancreatic tissue in in vitro studies [216]. Several studies have presented a correlation between EV infection and EV titers before seroconversion and the similar seasonality of EV infection and seroconversion[218,219]. Of particular interest is the phenomenon in which an initial EV infection spreads to the pancreatic islet with a subsequent inflammatory reaction. Certain predisposed individuals then fail to clear the infection, leading to the persistence of the virus in the β-cells in a chronic infection, during which the

virus slowly replicates and produces viral DNA. This persistence, in turn, stimulates innate immunity and drives inflammation and autoimmunity[220].

In addition to EV infection, several other viral infections have been implicated in T1D risk. Among these are respiratory and intestinal infections, such as rotavirus and cytomegalovirus[221,222]. Data from microbiome studies suggest an increased T1D risk in individuals with an abnormal microbiome, although few large-scale and long-term studies have been reported[223-225]. Additionally, early exposure to cereals[226,227], early introduction of cow's milk[228,229] and increased infant weight gain[230] have been proposed as potential T1D risk factors.

The countries with the largest increases in T1D incidence have experienced large improvements in sanitation and living standards. The so-called "hygiene hypothesis" postulates that decreased microbial pressure and a cleaner environment, coupled with a human immune system that has not had time to adapt, increase the risk of both allergy and autoimmunity[231]. However, it is likely that this is a multifactorial process that relies on additional environmental and genetic factors[232].

Higher omega 3 fatty acid intake has been reported to decrease diabetes risk in children, as has supplementation with probiotics during the child´s first month of life[233]. Breast feeding has shown conflicting results, with several studies seeing no correlation with T1D risk[234] and some studies showing a protective effect in some subsets of subjects[235].

Promoters of progression

Several factors that accelerate the progression to T1D have been identified. Conflicting evidence exists regarding the effect of nitrite/nitrate intake and progression to T1D[236,237]. Increased growth, in terms of both length and weight, as well as puberty, increases the demands on β-cells and induces insulin resistance in some instances. Accelerated progression to T1D has been described for patients with increased weight gain, as well as increased weight and length[238,239]. Some data also suggest that increased intake of carbohydrates will accelerate progress from stage 2 to stage 3 of T1D[240].

At the present time, no protective factors that slow the progression to T1D have been described.

Prediction of T1D

Early Prediction of T1D risk

Screening of newborns and very young children was originally accomplished using heredity data from first-degree relatives. We now know that 85-90% of newly diagnosed T1D patients lack a first-degree relative with the disease; thus, this approach misses far too many potential subjects[241]. The use of HLA genotype data has provided a cost-effective and rapid screening process. However, since only approximately 50% of the genetic risk is conferred by HLA, a fairly large proportion of risk information has been missed by this approach[156]. The utility of enhanced, high-resolution HLA genotyping with next-generation sequencing has recently been demonstrated, with a specificity and sensitivity of 90% and the ability to identify 80% of those with a lifetime risk of T1D[242].

Several studies have now attempted to improve early T1D prediction by using previously identified T1D-related SNPs[243]. The TEDDY study demonstrated that PTPN22, ERBB3, SH2B3 and INS correlate with the risk of developing IA in TEDDY children. The presence of PTPN22 and ERBB3 was later shown to confer an increased risk for GADA only or IAA only as the first islet autoantibody, while the INS SNP was shown to protect against IAA only and a polymorphism in BACH2 was associated with an increased risk of GADA only[44]. Similarly, in the BABYDIAB cohort, SNPs in IFIH1, CTLA4, PTPN22, IL18RAP, SH2B3, KIAA0350, COBL and ERBB3 were identified as candidates to increase the precision of T1D risk assessment[244]. In the DAISY study, the use of additional SNPs improved prediction when used together with the HLA genotype. PTPN22 and UBASH3A SNPs were associated with IA development and INS, UBASH3A and IFIH1 were associated with progression to T1D[188]. These findings were later tested in a genetic risk model that included three or ten SNPs in an effort to further improve diabetes prediction, with possible improved prediction in the general population[185].

A TrialNet study also reported results that support the improved prediction of IA when using non-HLA genetic markers, with GLIS3 and SH2B3 improving risk prediction in comparison to HLA alone, as well as CTLA4 for predicting progression from single to multiple autoantibodies[187].

Prediction of T1D risk

The presence of islet autoantibodies has added significant accuracy to the prediction of T1D risk and also represents the earliest sign of β-cell autoimmunity. It has been reported that a single autoantibody increases the T1D risk from 0.4% to

14.5% in at-risk children, but the reversion of islet autoantibody positivity also occurs[51,96]. With the occurrence of multiple islet autoantibodies, the risk is extremely high, with a 10-year risk of at least 70%[51]. The diabetes risk was also significantly higher for children with a higher number of islet autoantibodies. The utility of islet autoantibodies has been demonstrated repeatedly in both high-risk populations and the general population[245-248] (Figure 9.).

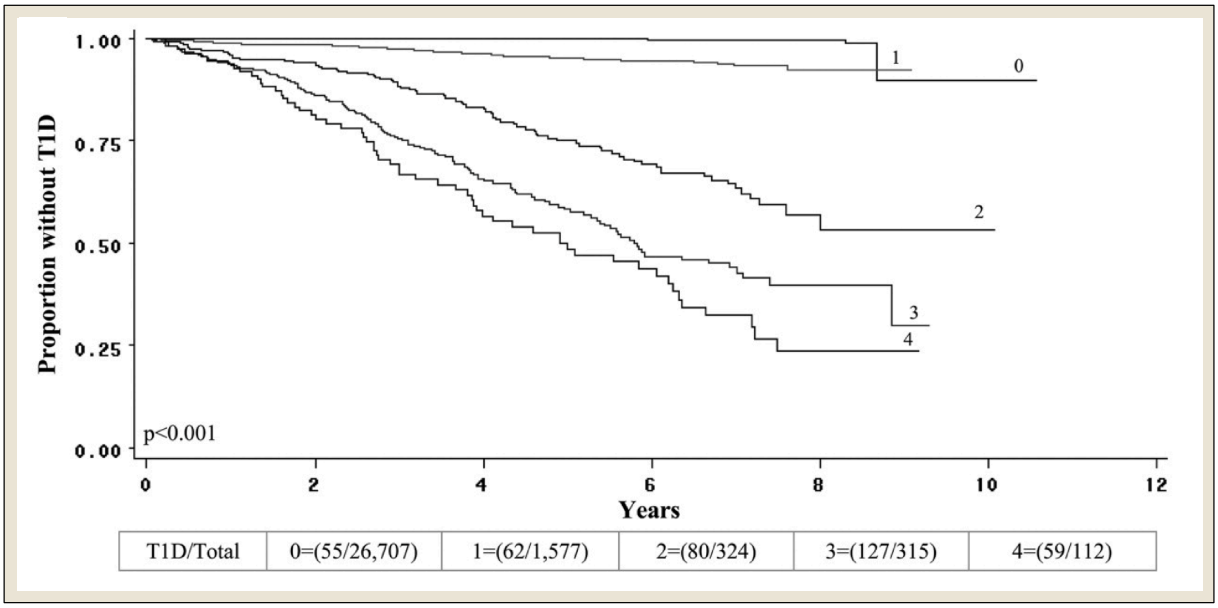

Figure 9. Progression to T1D in the DPT-1 cohort according to the number of positive islet autoantibodies. Numbers indicate number of positive autoantibodies; Fraction in parentheses shows the number who developed T1D among subjects at baseline. Reprinted with permission from Orban and colleagues[49].

Recent attempts have been made to increase the accuracy of T1D risk prediction by incorporating the autoantibody slope and titer[249] into T1D risk scores. The prognostic value of individual autoantibody titers, however, remains unclear[250].

Prediction of progression to diabetes

We know that a loss of glucose tolerance occurs months before the diagnosis of diabetes[251]. Several factors related to glucose metabolism can be used to predict the progression to dysglycemia and symptomatic T1D.

An intravenous glucose tolerance test (IvGTT) calculates the first phase insulin response (FPIR) as the sum of the serum insulin concentrations 1 and 3 minutes after the end of glucose infusion. A decline in the FPIR has been demonstrated during the last 1.5-2 years before diabetes diagnosis[252,253], and this decline may occur as early as 4-6 years before diagnosis[253]. A low FPIR (below the first percentile) has also been associated with a 50% risk of T1D within 1 year[254].

Oral glucose tolerance tests (OGTT) have also been shown to be of significant value in T1D prediction. In most studies, dysglycemia was defined as 30-, 60- or

90-minute glucose values ≧11.1 mol/L or 120-minute glucose values of 7.8-11.1. The C-peptide response during OGTT starts to differ from controls at least two years before diabetes diagnosis, with a decrease in the early C-peptide response. Conversely, the late C-peptide response increases, leading to a normal C-peptide AUC[255,256] (Figure 10). Significant declines in the peak C-peptide and the C-peptide AUC were only detected six months before diagnosis. Impaired glucose tolerance in the OGTT is associated with a HR above 8 for T1D diagnosis and a median time to diagnosis of 0.7 years[257].

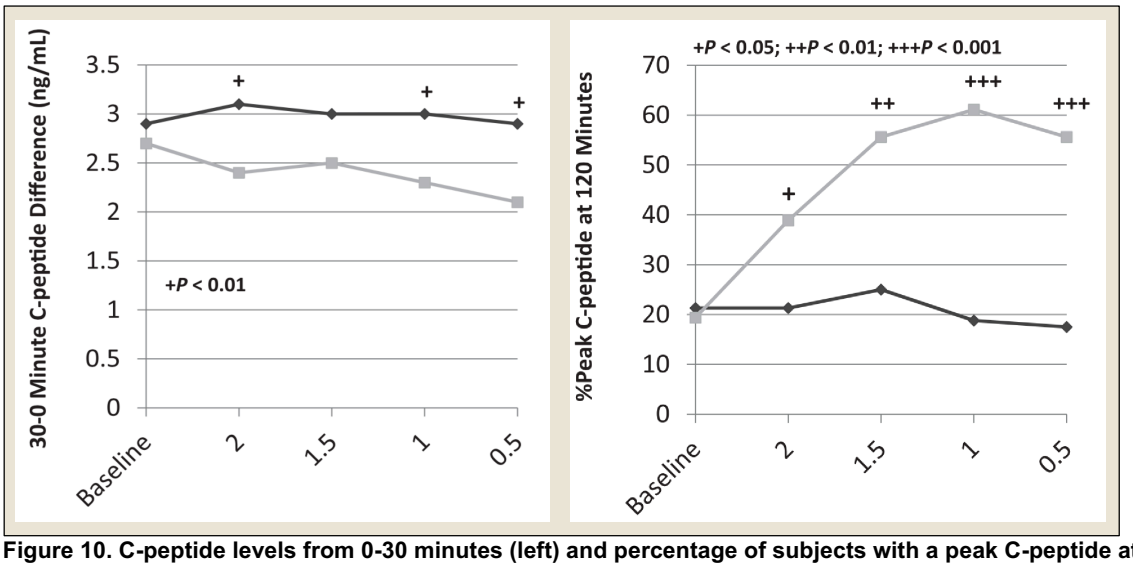

Figure 10. C-peptide levels from 0-30 minutes (left) and percentage of subjects with a peak C-peptide at 120 minutes (right) in the OGTT. X-axis shows years before diagnosis for progressors (grey line) or years before last visit (non-progressors). Progressors n=36; non-progressors n=80. Reprinted with permission from Sosenko and colleagues[251].

As a fairly late predictor of β-cell failure and T1D diagnosis, HbA1c has also proved to be a useful, simple and inexpensive tool. A slow increase in HbA1c, within the normal limits, has been described in multiple islet autoantibody-positive subjects[258]. In multiple antibody-positive children, a 10% increase in HbA1c was associated with an 84% 3-year risk of T1D and a HR of 5.7 in two large prospective studies[53,259]. HbA1c levels tend to be consistently higher in T1D progressors two years before diagnosis, and when HbA1c levels exceed 41 mmol/mol, the median time to diagnosis is 0.9 years[259].

Additionally, a simple random plasma glucose test can be of value for prediction. The DAISY study demonstrated the modest benefit of random plasma glucose, with a HR of 1.4 for T1D development[258]. In the DIPP study, a similar result was associated with a stronger risk for time to diabetes diagnosis[259].

With the development of continuous and flash glucose monitors, new possibilities for assessing glucose control and variability have emerged. Several studies are ongoing, and this technology will no doubt prove useful in prediction efforts.

Prevention of T1D

Several obstacles must be overcome before the goal of stopping the disease process of T1D has been achieved. Our understanding of the pathogenesis of the disease and the inciting and exacerbating agents involved in this process will hopefully be improved by ongoing studies, such as TEDDY, TrialNet[260], nPOD[87] and DiViD[261]. An acceptance of the concept of asymptomatic T1D will enable us to conduct studies before the majority of β-cells have been lost. Using this classification, with subjects at extremely high risk for progression, will also make prevention efforts easier to tolerate from an ethical perspective[262]. We must forego the mono-therapeutic approach and test approaches that minimize the side effects of immune suppression and improve prior studies´ lack of lasting effects. The possibilities for intervention are numerous and illustrated in figure 11. Collaborations on primary prevention trials are an exciting prospect, even more so as new targets for prevention are presented[263]. Finding a tolerable regimen that is suitable for the long-term treatment of young children will be a monumental task, as although current regimens have promising efficacy in some cases, they fail to provide acceptable side effects for general treatment.

Numerous studies have been performed with the aim of interrupting the T1D process, both before and after a clinical diagnosis of diabetes. An overview of the approaches that have been used to date comprises the remainder of this chapter.

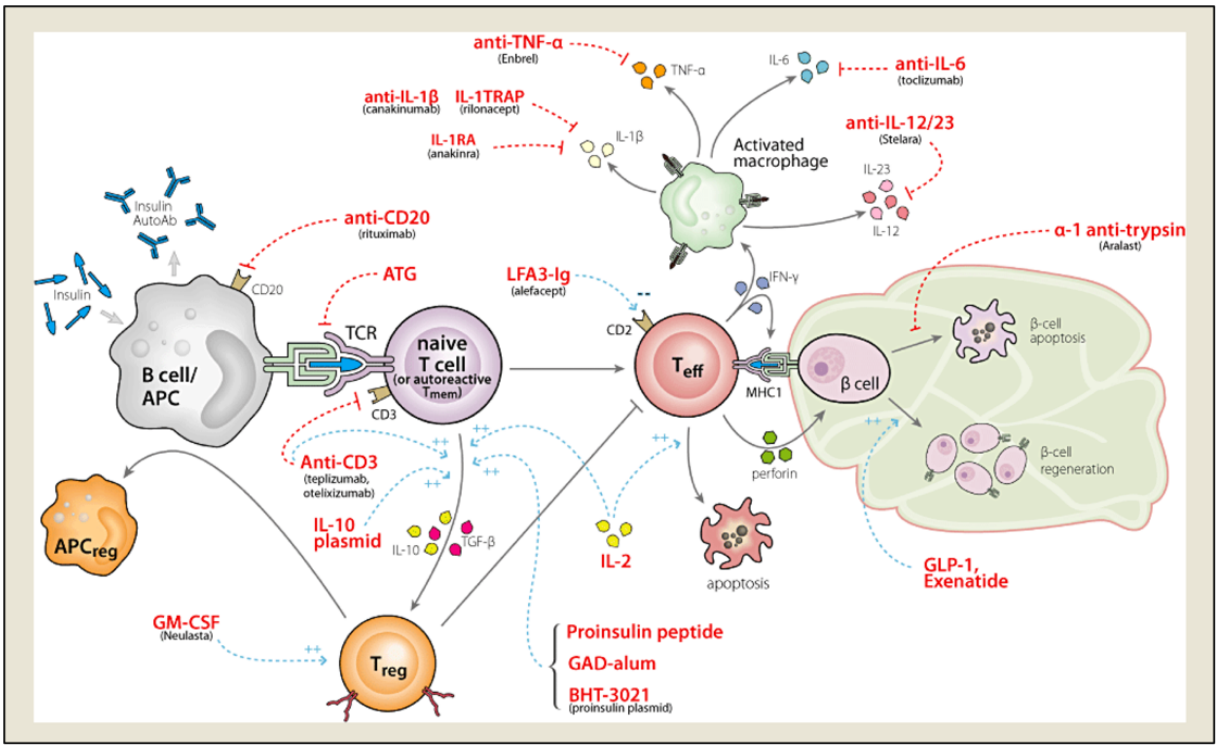

Figure 11. Pathways and opportunities to intervene in T1D. Some of the pathways and possible sites of intervention in T1D autoimmunity. Figure showing the pathways known to be involved in T1D etiopathology, as well as available drugs that affect these pathways. Reproduced with permission from Matthews and colleagues[264].

β-cell support

It has been hypothesized that β-cell stress leading to the increased release of antigens into the circulation could contribute to the autoimmune process of T1D and that over-worked β-cells lead to cellular dysfunction[265]. Attempts have been made to pharmacologically support the β-cells of at-risk or newly diagnosed T1D patients to delay the time to total insulin dependency.

Insulin

The DPT-1 study attempted to delay or prevent T1D diagnosis in first-degree relatives who were deemed to have a five-year risk of above 50% via the administration of subcutaneous insulin. No effects could be seen regarding the time to diagnosis or the cumulative incidence of diabetes between the groups[266].

Diazoxide

Attempts to achieve a resting period for the β-cells by blocking insulin secretion with Diazoxide have also been made. A short-term increase in insulin secretion was observed after Diazoxide treatment ended. Unfortunately, no differences in C-peptide AUC were observed between the treatment and placebo groups during follow-up[267].

β-cell protection

β-cells are known to have a lower capacity to cope with oxidative stress in comparison to the other cells of the endocrine pancreas. This limited anti-oxidative capacity and the ensuing vulnerability of β-cells have led to several attempts at protective treatments.

Vitamin D

Vitamin D has been reported to have an immune modulatory effect, shifting the immunological balance from a Th1 to Th2 response, as well as improving the ability of β-cells to withstand stress[268,269]. Epidemiologically, vitamin D has been linked to protection against T1D if patients are supplemented at birth or during the first year of life[212,270,271]. Maternal vitamin D levels during pregnancy have been inversely correlated with T1D risk[213]. However, when used as single therapy in new-onset T1D patients, this treatment has not been successful in delaying or preventing diagnosis or preserving insulin secretion[272,273].

Anti-oxidants

As an anti-oxidative agent, nicotinamide has been thought to be able to protect β-cells from damage. However, clinical trials have demonstrated no efficacy in preventing T1D clinical onset[274]. Other attempts at mono-therapy with anti-oxidants have also failed to show effects in humans, despite promising animal studies[275].

Immune modulation

BCG vaccination

Vaccination with Bacillus Calmette-Guerin (BCG) has been used in early studies as an immune modulator. In theory, vaccination would increase the host's TNF-α levels and reduce the levels of autoreactive T-cells. However, clinical trials have demonstrated no beneficial effect[276]. In addition, epidemiological studies have reported no correlation between T1D incidence and vaccination status[277].

Anti-CD3

CD3, which is a co-receptor for the T-cell receptor (TCR), is involved in the activation and differentiation of naive T-cells into pathogenic effector T-Cells (T_{eff}). CD3 prevents activation and promotes the depletion of T-cells, with Teffs being more sensitive than regulatory T-cells (T_{reg}). This process leads to the depletion of T_{eff} and the restoration of the T_{eff}/T_{reg} ratio.

Using anti-CD3 monoclonal antibodies (Oxelizumab, Teplizumab), several large studies have been performed, with conflicting but promising results. Oxelizumab has been shown to preserve insulin secretion in new-onset T1D patients. However, a lower dose failed to preserve insulin secretion in new-onset patients[278-280].

Co-stimulation blockade

Abatacept is a CTLA-4 receptor blocker fused to a modified fc-portion from human IgG1. This drug acts as decoy receptor for cd80/86 and blocks CD28-induced co-stimulation, interfering with T-cell activation, proliferation and differentiation. In a TrialNet study, new-onset T1D patients received 27 infusions of Abatacept or placebo over two years. A decreased rate of C-peptide decline and a higher C-peptide AUC were seen in the treatment group. Unfortunately, the autoimmune process only seems to be delayed by the treatment[281].

B-lymphocyte-targeting approaches

B-cells are involved in both antigen presentation and T-cell activation. On the B-cell membrane, CD20 is required for B-cell activation and

proliferation. Rituximab, which is a monoclonal antibody to CD20, was used in a TrialNet study in which a four-dose course was given to new-onset T1D patients (8-45 years old). The mean C-peptide AUC at year 1 was higher for the treatment group. The treatment group also had a lower HbA1c and required fewer insulin doses[282]. However, at 30 months post-randomization, there was no difference in the decline of the AUC C-peptide between the treatment groups[283]. The lack of long-term effect may be due to rapid natural B-cell replenishment; thus, additional therapeutic agents may be required to achieve a long-lasting immunological effect.

Cytokine-based targets

A dysregulation in the balance between regulatory T_{reg} cells and T_{eff} cells has been suspected to be central to autoimmunity. Cytokines, such as IL-2, regulate both the function and size of the T-cell population. The IL-2 receptor on T_{reg} cells has a higher affinity and is more numerous. Thus, T_{reg} cells respond to a lower concentration of IL-2 than T_{eff} cells. This concept has been used in several studies to treat the autoimmune process and has been proven to prevent T1D and reverse established disease in mice[13]. IL-2 has been used without significant adverse events and with a dose-dependent effect on T_{reg} cells in a phase I study[284]. Phase II studies are ongoing in new-onset T1D patients but have yet to reach their endpoints. IL-23 and IL-12 are both involved in the amplification of pro-inflammatory pathways. Ustekinumab targets the shared p40 subunit of IL-12 and IL-23, blocking differentiation and central immunological signaling.

Two studies are currently active. One phase 1 study involves new-onset T1D patients (18-35 years) and ends in 2016. A second study uses a combination of Ustekinumab and islet neogenesis-associated protein (INGAP)-peptide in established T1D patients (19-40 years)[14].

Tyrosine kinase inhibitors

The tyrosine kinase inhibitors Imatinib and Sumatinib have previously been shown to preserve β-cell function in NOD mice, possibly via the platelet-derived growth factor receptor (pdgfr)[15]. Only case report evidence exists regarding the effects of these inhibitors in humans. A phase II study in patients with new-onset T1D is currently in progress.

T-regulatory cell-based therapy

T_{reg}-cells inhibit T_{eff}-cells and regulate autoimmunity and immune tolerance. A low number of T_{reg}-cells and impaired T_{reg} cell function have been observed among T1D patients[16]. T_{reg}-cells that are expanded ex vivo and then infused back into the patients have been investigated in studies that reported higher C-peptide levels in the treatment groups.

Ex vivo-expanded T_{reg}-cells have shown good suppressive function in in vitro studies and a good safety profile in a phase 1 study[18]. In children with new-onset T1D, the autologous infusion of ex vivo-expanded T_{reg}-cells prolonged remission, with higher C-peptide levels than the non-treated group[17]. A dosage study and a combination trial with polyclonal T_{reg}-cells and IL-2 have been started[19].

Immune Suppression

Corticosteroids

Corticosteroids have proved to be extremely valuable in many autoimmune diseases. Unfortunately, because corticosteroids induce insulin resistance, they are not suitable for the long-term treatment of T1D autoimmunity. Few studies have been performed. The results showed possible immune effects and conflicting efficacy regarding the ability to preserve insulin secretion[285,286].

Cyclosporin

Cyclosporin treatment was one of the first attempts to stop the immune process of T1D. This was achieved, however, with a significant risk of side effects related to immune suppression and renal damage[287,288]. Studies using lower doses of Cyclosporin have shown greater safety but at the cost of a reduced effect[289].

Azathioprine

Azathioprine, as a single treatment, has been used in previous studies. The initial results regarding remission were promising but failed to achieve effects beyond the initial period after diagnosis[290-292].

Anti-Thymocyte globulin

Anti-thymocyte globulin antibodies against human T-cells have also shown promising effects on remission but failed to achieve long-term effects as a single therapy[293].

Antigen-based therapies

Reverse vaccination, which is similar to the ASIT concept used in allergology, involves repeated doses of exogenous autoantigens that are given to achieve a long-term effect on regulatory, non-inflammatory T-cell responses. The goal is to induce immunological tolerance to the antigen via T-cell anergy, as well as the induction of T_{reg}-cells[294].

Insulin

In the DPT-1 study, 372 subjects with a five-year T1D risk of 26-50% received oral insulin in a double-blind, placebo-controlled study. No effect could be seen on diabetes rates in the total cohort. However, a possible effect could be seen in subjects with insulin autoantibodies[295]. The results of a follow-up TrialNet study of oral insulin, which was closed in 2016, are still pending publication. In the Pre-Point study, the protective effects of high-dose oral insulin in children (2-7 years old) with a HLA risk genotype were studied, with potential effects on T_{reg}-cells for children receiving 67.5 mg of oral insulin daily[296]. An active small, open-label TrialNet study is being conducted to assess the effects of dosing and schedules of oral insulin. Nasal insulin has also been used, but the treatment failed to delay or prevent T1D[297].

Pro-insulin peptide

As an immunologically active but non-functional agent, the pro-insulin peptide has been used to achieve immune tolerance. In a phase I study, the peptide was demonstrated to be safe and to have possible positive immunological effects[298]. A recent study also demonstrated the safety of the concept and reported promising results regarding the preservation of insulin secretion[299].

GAD

As one of the major autoantigens in T1D, GAD is an interesting target for immune modulation therapy. Early studies in mice yielded promising results regarding both safety and efficacy[300-302]. Safety was established in a phase I study, as well as later phase II/III studies. A potential effect on C-peptide preservation in adult LADA patients and safety information have also been published[303,304]. In children with new-onset disease, GAD was also demonstrated to be safe and to significantly preserve C-peptide in children with a shorter disease duration, but no effect was reported in other studies[305-307].

Combination therapies

Given the disappointing results of many potent therapeutic agents in single therapy trials, especially with respect to long-lasting effects and the avoidance of adverse events, combination therapy may be the only way forward.

In a phase I/II study, high-dose immunosuppression combined with autologous stem cell transplantation is being used to induce tolerance in new-onset T1D patients (13-31 years). Cyclophosphamide and G-CSF are used to harvest stem cells and later re-infuse them after conditioning with Cyclophosphamide and anti-thymocyte globulin. During 30 months of follow-up, C-peptide was increased, and the majority of the patients were insulin-free or required only low-dose insulin[308].

Other studies investigated similar regimens with beneficial results on insulin dependency six months after treatment and 4 years after treatment[309]. However, treatment is associated with significant side effects, with 52% of subjects experiencing adverse effects and one death caused by immune suppression and sepsis.

Protocols are being altered to lower the risks of immune suppression, including a lower dose of anti-thymocyte globulin and prolonged treatment with G-CSF. Using this regimen, a majority of treated subjects showed no decline in β-cell function 12 months after treatment and exhibited an improved T_{reg}/Teff ratio. Side effects occurred at more acceptable levels and were reversible[310].

Aims of the thesis

This thesis focuses parts of the process of T1D research in a pediatric setting, from risk factor identification through follow-up and intervention attempts.

The specific aims of this thesis were to:

- Investigate the effect of islet autoantibodies in umbilical cord blood and the risk of T1D in the DiPiS cohort. (Paper I)
- Evaluate the possible effects of early life stress and severe life events on the risk of T1D in the DiPiS cohort. (Paper II)
- Examine the effects of exposure to analgesic antipyretics during the first two and a half years of life on the development of IA at age six years in the TEDDY cohort and describe the differences in use between the respective sites. (Paper III)
- Describe the outcomes of the children who had been enrolled in DiPiS at T1D diagnosis with respect to autoantibody status, metabolic derangement and peri-diagnostic morbidity. (Paper IV)
- Examine the outcomes of children who were previously enrolled in the DiPiS study follow-up after the diagnosis of T1D in comparison to those of children who were diagnosed from the general population. (Paper IV)
- Investigate whether GAD-Alum, given as two subcutaneous injections, is safe or causes severe adverse events in children with a high risk of T1D. (Paper V)
- Determine if GAD-Alum delays the time until T1D diagnosis. (Paper V)

Study Populations

The DiPiS Study

The DiPiS (Diabetes Prediction in Skåne) study is a prospective follow-up study in at-risk children. Children with a primarily increased genetic risk of T1D are followed until the age of 15 years. The aim of the study is to identify environmental risk factors for T1D and improve diabetes prediction.

Between September 2000 and August 2004, 48,058 children were born in the five participating hospitals in Skåne, which is in the southern part of Sweden. After obtaining oral consent from the mothers, 35,683 umbilical cord blood samples were collected for HLA-DQ and cord blood autoantibody analysis. When the child was two months old, the parents were invited to participate in the study and complete a questionnaire to collect demographic data, family medical history of interest and data concerning events and illnesses from pregnancy until two years of age. The parents of 25,378 children completed the questionnaire and gave written consent to participate in the study. A basic risk score was constructed based on the following parameters:

- HLA-DQ genotype (DQ2/8, DQ8/8 or DQ8/X, DQ2/2 or DQ2/X or DQX/X (X is neither 8 nor 2))
- Presence of maternal infections during pregnancy
- High or low relative birth weight
- First-degree relative with insulin-dependent diabetes
- Positive for umbilical cord autoantibodies

Using this risk score, 7,826 children were offered yearly follow-up with a questionnaire and sampling for islet cell autoantibodies. In total, 3,889 parents consented to follow-up and entered the main DiPiS cohort (Figure 12.) At the time of the planning of the study, we presumed that very few children would develop diabetes during the first years of life, so follow-up of the children started at age two years.

The participants are sampled yearly for islet autoantibodies and asked to return a questionnaire covering the last year in the child´s life. Children who seroconverted to two or more islet autoantibodies were offered more intense follow-up every 3 months, which included islet autoantibodies, random plasma

glucose, HbA1c, growth parameters and a yearly oral glucose tolerance test (OGTT)

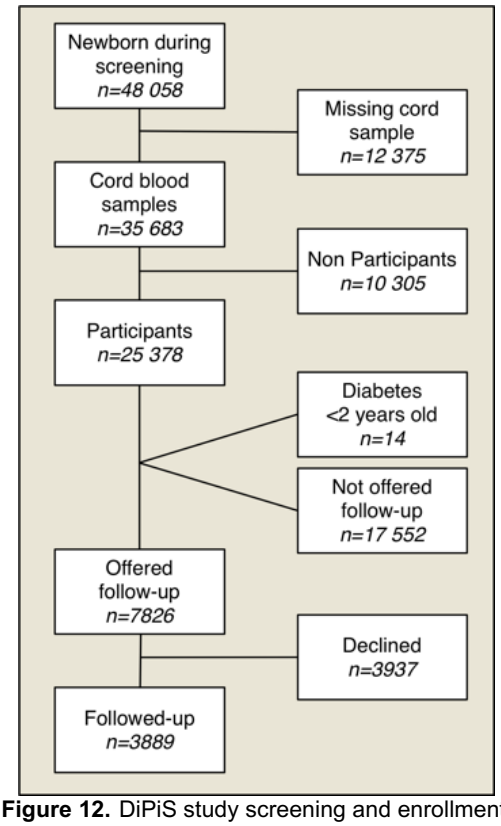

Figure 12. DiPiS study screening and enrollment.

As of August 31, 2017, a total of 222 children from the original birth cohort of 35,683 had developed T1D. Before follow-up began at two years of age, 14 children had been diagnosed. In the cohort that consented to prospective follow-up, 130 children had been diagnosed with T1D and 88 multiple autoantibody positive children were in active, intense follow-up (Figure 13.).

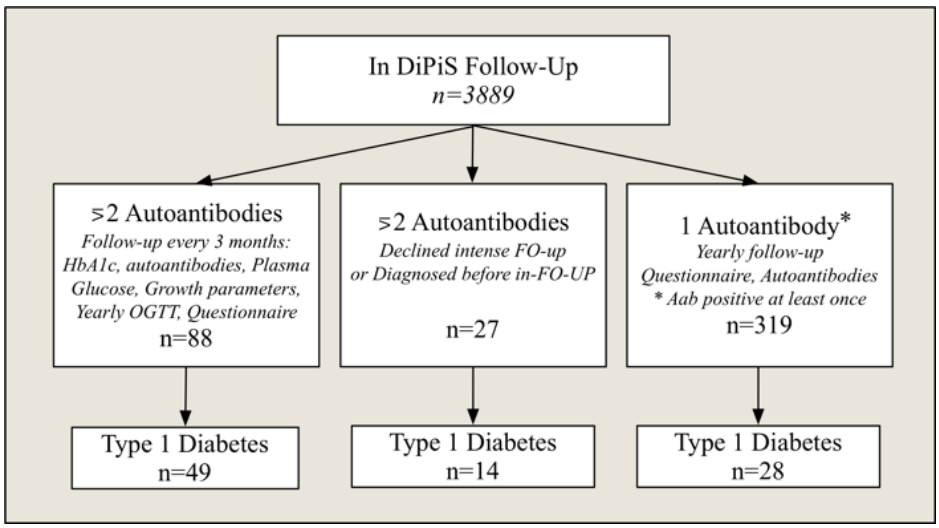

Figure 13. Flow chart of the children diagnosed with T1D in DiPiS.

The TEDDY Study

The environmental determinants of diabetes in the young study (TEDDY) is a prospective cohort study that aims to find environmental factors that influence the development of IA and T1D in children. The study includes six clinical research centers, including three in the US (Colorado, Georgia/Florida, Washington) and three in Europe (Finland, Germany, and Sweden)[74] (Figure 14.)

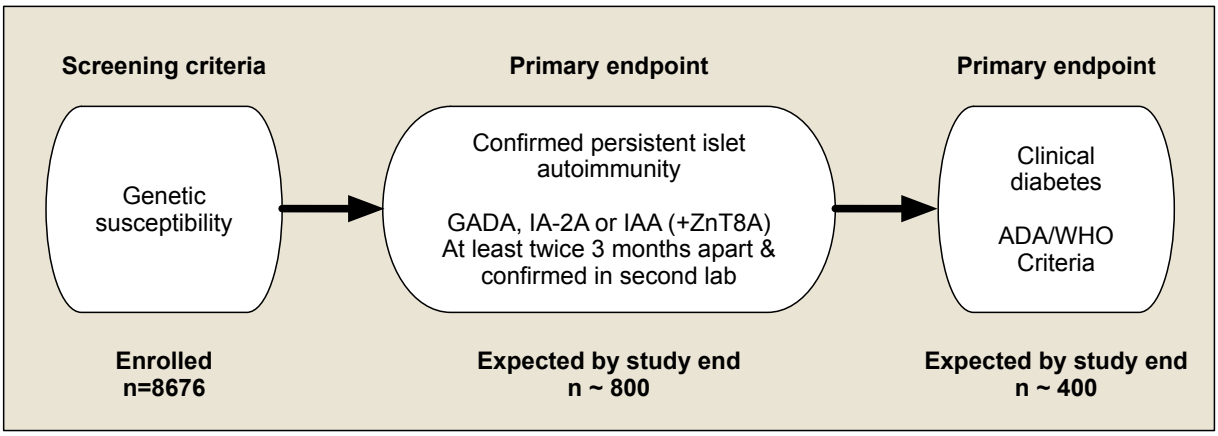

Figure 14. Children enrolled in the TEDDY study, primary study endpoints and expected children at each endpoint by study completion. ZnT8A is only analyzed if any of the other autoantibodies are positive.

From September 2004 to February 2010, TEDDY screened 434,620 children, of whom 21,321 were eligible for follow-up. Screening was performed at TEDDY´s six clinical centers. Eligibility was determined using separate genetic HLA DQ criteria for children from the general population and children with a first-degree relative with T1D[311].

Out of the eligible children, 8,676 children were enrolled in intensive follow-up. Follow-up started at age 3 months and included site visits every 3 months until the age of 4 years. After that, visits are performed every six months for autoantibody-negative children and every three months for children who are islet autoantibody-positive. A plethora of data are collected according to the study plan, including blood for islet autoantibodies, PBMC, DNA/mRNA, HbA1c and storage, urine, nasal swabs, nail clippings, tap water, salivary cortisol, accelerometer data, body composition data, etc. Interviews are performed at each visit to account for infection, medication and immunization data, food records, negative life events, parental anxiety and depression and physical activity[312].

All samples are kept in a central repository, and all data are kept in the study data coordinating center in Tampa, Florida.

The DiAPREV-IT study

Diabetes prevention immune tolerance, DiAPREV-IT, is an investigator-initiated, randomized, double-blind, placebo-controlled clinical trial (EuCT 2008-007484-16, NCT01122446). The primary aim of the study is to investigate the safety of GAD-Alum in children at risk for T1D. The second aim is to evaluate if immune tolerance treatment with GAD-Alum can delay and prevent the autoimmune process that leads to a diagnosis of T1D.

Study drug

The study drug for DiAPREV-IT, Diamyd® (GAD-Alum), was previously tested in several studies. However, none of those studies included children who were at high risk of T1D but not yet diagnosed. Previous studies of GAD-Alum have indicated effects on the progression of IA in spontaneously diabetic non-obese diabetic (NOD) mice[302,313-316]. A phase IIa study in patients with latent autoimmune diabetes in adults (LADA) demonstrated safety and potential efficacy in the group of patients receiving GAD-Alum[303]. Another phase IIb study was unfortunately invalidated due to identification issues. However, no concerns regarding safety were raised during the 18-month study period.

The potential efficacy of GAD-Alum in children has also been presented in a phase II study in recently (<18 months) diagnosed children with T1D. This study demonstrated both a favorable safety profile and an effect on the preservation of β-cell function after 30 months[305]. Further, a 3-armed phase III study in children with a recent onset of T1D (<3 months) has been published. In this study, one study arm received GAD-Alum as two single doses and a second study arm received two additional single doses on days 90 and 270 to potentially optimize the treatment. This study did not fulfill its efficacy goals after 15 months of follow-up and was closed early. However, no safety concerns were raised in the study[307].Additionally, a three-armed TrialNET study on 145 new-onset patients (3-45 years) with either 3 doses of GAD-Alum, 2 doses of GAD-Alum or Placebo filed to demonstrate efficacy on preservation of insulin secretion at 1 year after treatment[306].

Study inclusion

Children with positivity to GADA and at least one more islet autoantibody were recruited to the study. After consent and randomization, the participants received 20 µg of GAD-Alum in a prime-and-boost regimen on days 1 and 30.

Children with ongoing IA were recruited from the DiPiS study (described above), the TEDDY study (described above) and from the subjects followed in the Trial-Net natural history study (TN01). Trial-net participants were only invited when no TrialNet prevention study was available or suitable for the child. The inclusion/exclusion criteria can be seen below.

Inclusion criteria

- Aged 4-17.99 years
- Positive for GAD autoantibodies
- Positive for at least one more islet autoantibody (IAA, IA-2A or ZnT8R/W/Q/A)
- Absence of the protective HLA DQ6 genotype

Exclusion criteria

- Diabetes diagnosis
- Ongoing treatment with immunosuppressant therapy
- Significant abnormal hematology results at screening
- History of acute reaction to vaccines
- History of epilepsy, serious head trauma or cerebrovascular accident or clinical features of continuous motor unit activity in proximal muscles
- Participation in other clinical trials the last 3 months
- Significant illness other than diabetes within 2 weeks prior to first dosing
- HIV or hepatitis
- Other serious disease or condition precluding subcutaneous injection

Study participants were recruited between April 2009 and January 2012. After baseline staging with both IvGTT and OGTT on two separate visits, the children were randomized to receive either GAD-Alum (Diamyd®, Diamyd Medical, Sweden) (n=25) or placebo (n=25). The study participants were then followed for five years, with visits every 3 months. Follow-up included physical examination, hematology, cellular immunological analyses, islet autoantibodies, glucose tolerance tests, HbA1c and thyroid and celiac autoantibodies.

For the diagnosis of diabetes, the criteria adopted by the WHO and the ADA were used[24,25].

Laboratory Methods

HLA Genotyping

HLA genotyping in the DiPiS study

HLA genotyping was performed on dried blood spots (DBS) on filter paper, as originally described by Ilonen and Sjöros[317-319]. For the analyses in this thesis, HLA was classified as HLA-DQA1*0501-DQB1*0201 (DQ2) or HLA-DQA* 0301-DQB1*0302 (DQ8) and stratified into four risk groups: DQ 2/8, DQ8/8 or 8/X, DQ2/2, or 2/X or DQ X/X (X is neither DQ2 nor DQ8).

HLA genotyping in the TEDDY study

The TEDDY screening system that involves HLA-based mass screening for T1D risk has been thoroughly described previously[320].

Genotype screening is performed using either a dried blood spot (DBS) punch or a small-volume whole blood lysate (WBL) specimen format[321]. The screening blood sample is typically obtained at birth as a cord blood sample, but potential participants, especially first-degree relatives of T1DM patients, can be screened using a heel stick capillary sample up to the age of 4 months. After PCR amplification of exon 2 of the HLA Class II gene (DRB1, DQA1 or DQB1), alleles are identified by direct sequencing, oligonucleotide probe hybridization, or other genotyping techniques. Additional typing to sufficiently identify certain DR-DQ haplotypes is specified for each clinical center.

Confirmation of the HLA genotypes is performed by the central HLA Reference Laboratory at Roche Molecular Systems in Oakland, CA (Dr. Henry Erlich) on 100% of the eligible subjects at 9 months of age. This process includes high-resolution genotyping of HLA-DRB1, DQA1, and DQB1. If a subject is not confirmed to be HLA eligible, a detailed explanation is provided to the parents, together with the option of continuing in TEDDY. These non-eligible subjects that stay in follow-up are noted in the databases maintained by the DCC. The HLA Central Laboratory genotypes the DNA sample obtained at 9 months of age and also evaluates three non-HLA markers of T1D genetic susceptibility: insulin 5'-

VNTR using the –23 Hph polymorphism, the CTLA-4 A49G polymorphism in exon 1 and the PTPN R620W polymorphism. Genotyping for HLA- DPB1, HLA-A, HLA-B, MIC-A and/or other MHC and non-MHC markers may occur in the future, as determined by the study group[311].

Islet autoantibody analysis

Islet autoantibody analysis in the DiPiS and DiAPREV-IT studies

Autoantibodies to GAD65 and IA-2: Analysis in dried blood spots, serum or plasma

GAD65 autoantibodies (GADA) and IA-2 autoantibodies (IA2A) were analyzed in dried blood spots (DBS) with a radioligand binding assay (RBA)[322]. GADA and IA2A levels were expressed as units per mL (U/mL) derived from the WHO standard 97/550. The samples were considered to be positive if the IA2A levels were above 5 U/mL or the GADA levels were above 34 U/mL.

Autoantibodies to IAA

Noncompetitive method: Serum samples (7 mL) were added to duplicate wells of a 96-well microplate, and 36 mL of 125I insulin (30) with an activity of 60,000 cpm/well was added, then incubated on a shaker at 4°C for 48 h. PAS in a 40% slurry (50 mL) was added to a filter plate and washed three times with 200 mL of Tris buffer using a Micro-Plate Strip Washer (BioTek ELx50; BioTek Instruments, Bedfordshire, U.K.). Supermix scintillation solution (50 mL) was added to the wells after the plate had dried for 15 min. The radioactivity was measured in a ß-counter (Wallac Micro Beta Trilux; PerkinElmer).

Competitive method: Positive samples for IAA were further analyzed using a competitive method. Serum samples (7 mL) were added to four wells in a 96-well plate. To these wells, 36 mL of 125I insulin with an activity of 60,000 cpm/well were added, with 0.072 IU (or 2 IU/mL) of unlabeled insulin (Actrapid; Novo Nordisk) added to the last two wells. The plates were incubated and examined under conditions similar to those described for the noncompetitive method. IAA levels were calculated as relative units and were related to positive controls. Positivity for IAA was set to 1.9 relative units. The competitive method was used to verify false-positive binding in the noncompetitive assay. However, in subsequent analysis, the competitive assay was used.

Autoantibodies to ZnT8

ZnT8 autoantibodies (ZnT8A) were analyzed in 5 μL of serum with RBA, as described previously[323]. Duplicate samples were incubated with equal amounts of the three radio-labelled ZnT8 R/W/Q variants. Every putative positive sample (cut-off >59 U/mL) was analyzed for each ZnT8 variant separately.

Islet autoantibody analysis in the TEDDY Study

Islet autoantibodies to insulin, GAD65 and IA-2 were measured in two laboratories by RBA[128,324]. In the U.S., all sera were assayed at the Barbara Davis Center for Childhood Diabetes at the University of Colorado Denver; in Europe, all sera were assayed at the University of Bristol, the U.K. Both laboratories have previously shown high sensitivity and specificity, as well as concordance[119]. All samples positive for islet autoantibodies and 5% of negative samples were re-tested in the other reference laboratory, and the results were deemed to be confirmed if concordant[312].

To optimize concordance, harmonized assays for GADA and IA-2A replaced previous assays, in January 2010[128]. Based on a receiver-operator curve analysis, prior samples that needed to be re-analyzed with the harmonized assays included: Denver GADA between -0.015 and 0.042; Bristol GADA between 10.69 and 36.72; Denver IA-2A between -0.004 and 0.016; and Bristol IA-2A between 6.69 and 10.58.

Definition of IA in the TEDDY study

In the TEDDY study, persistent IA is defined as being positive to IAA, GADA or IA-2A, with the results confirmed at both laboratories and on at least two consecutive visits. The date of persistent autoimmunity was defined as the draw date of the first sample of the two consecutive samples which deemed the child as persistently positive for a specific autoantibody (or any autoantibody).

To exclude children who were born with maternal islet autoantibodies, the islet autoantibody status of the mother was assessed when the child was 9 months. If a child was islet autoantibody-positive under the age of 18 months, their autoantibody status was set as “pending.” Autoantibody sampling was performed every three months until 18 months of age. If maternal autoantibodies were present, the child was not deemed to be persistently islet autoantibody-positive unless they had a negative sample prior to their first positive sample.

Statistical methods

Paper I

Study population and data acquisition

Paper I focus on prenatal risk factors for T1D in the DiPiS cohort. We used baseline demographic data collected at age 2 years from all parents who returned the questionnaire on psychosocial, medical and hereditary factors (Figure 15.). Prevalence data regarding T1D status were retrieved similarly to paper I from local pediatricians and via the BDD study. Umbilical cord blood islet autoantibodies were analyzed in DBS eluates, as described earlier in this thesis. The present study only includes cases of T1D, and all other types of diabetes were excluded from the analysis.

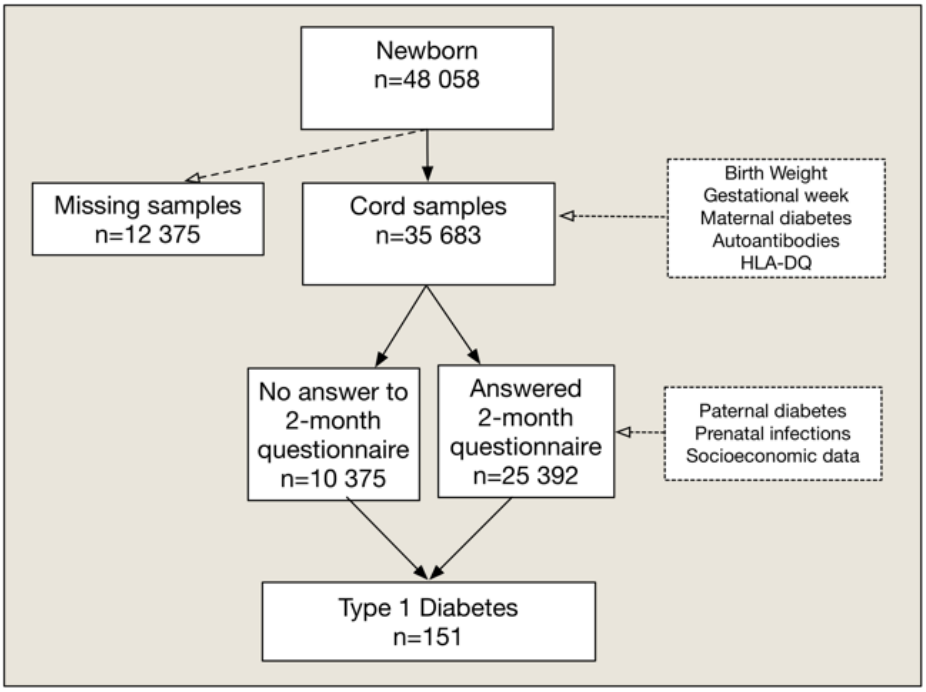

Figure 15. Participant selection for Paper II.
Figure also including number of answered questionnaires at 2 months and 2 years.

Statistical methods

Potential significant risk factors for T1D were identified using a univariate Cox proportional hazards model. A multivariate model Cox proportional hazards model

was used to test for independent associations for each autoantibody, birth weight, gestational length and infections. The model was stratified for HLA-DQ risk and also adjusted for gender, maternal age, and parental T1D. Hazard ratios for HLA-DQ risk groups were calculated in a model without stratification, and a separate baseline hazard for HLA genotype was fitted for each group. The variables available as possible independent predictors were autoantibody status in umbilical cord blood for IA-2, insulin and GAD (deemed positive if above the 95th percentile and treated as categorical variables), gestational week (<37, 37-39, 40-41, >41), the relative birth weight percentile, and infection during pregnancy (yes/no). As possible confounders in the analysis, HLA risk group (DQ X/X, DQ2/2 or 2/X, DQ 8/8 or 8/X, DQ 2/8, X is neither 2 nor 8), gender, parental T1D and mother's age at delivery were available.

Statistical analyses were performed using SPSS version 21 (SPSS, Chicago, IL) and R version 3.03, using the survival package (R Core Team (2014). R Foundation for Statistical Computing, Vienna, Austria.).

Paper II

Study population and data acquisition

In paper II, we focus on the possible effects of early life stress on T1D risk. When the children sampled with umbilical cord samples were two months old, a two-part questionnaire was sent out, with one part focusing on prenatal and antenatal events, as well as baseline demographic data on the parents, and the other part focusing on hereditary data on diseases in the family. A total of 23,187 (65%) questionnaires were returned to the study. The children who were invited to follow-up were then asked to complete a more thorough questionnaire regarding the child´s first two years of life. The questions covered a wide range of fields, including socioeconomic data, support system, depression and anxiety related to parenting, introduction of food, infections and medications. This "Two-year questionnaire" was answered by 3,861 families (49%). The analysis in this study uses data from both the two-month and the two-year questionnaires. The study selection can be seen in Figure 16. T1D incidence was reported by local pediatricians, as well as via the Better Diabetes Diagnosis study (BDD), with excellent coverage across Sweden[134]. All children with answers to the two-month and two-year questionnaires were included in the study.

Severe life events

On the two-month questionnaire, the family answered questions regarding severe life events during or after pregnancy. The overall question of "Did you experience anything that you would consider to be a severe life event during pregnancy or

after the child´s birth?" was followed by eight choices with tick-boxes for "Death of close relative"; "Severe disease in the family"; "Serious accident in family"; "Own divorce or separation"; "Been subjected to violence"; "Lost employment"; "Husband/Partner lost employment"; and "Serious conflict with the child´s father or another significant person," as well as tick-boxes to indicate if the event took place during or after pregnancy. These questions were analyzed regarding T1D risk, both as a joint variable indicating that an event of any kind had taken place during or after pregnancy and as individual events.

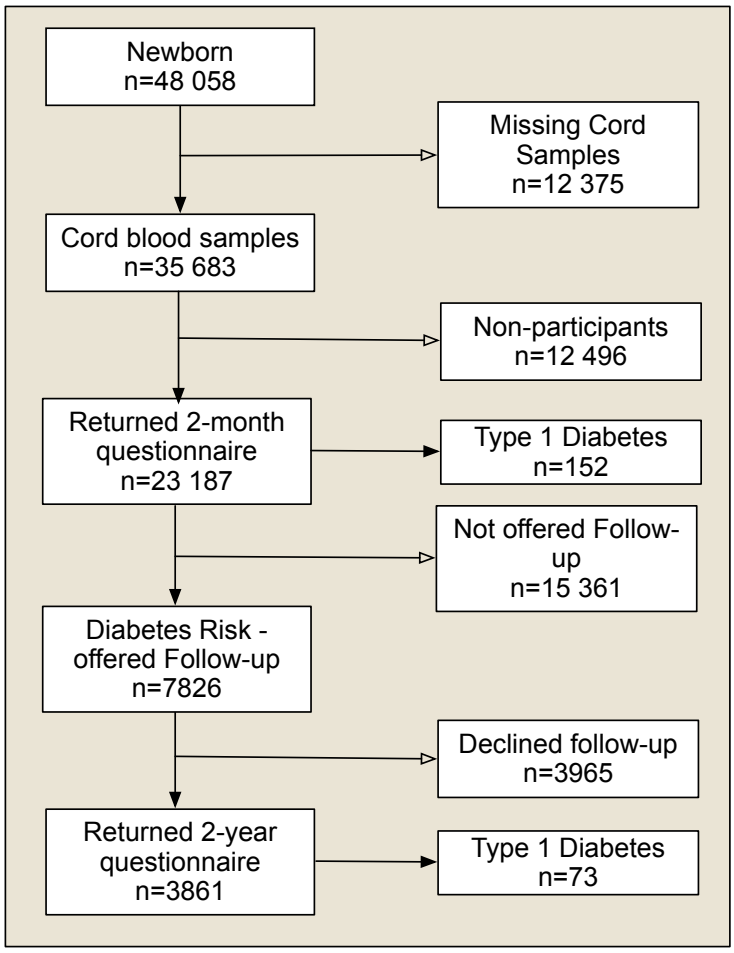

Figure 16. Flow chart of Study participation in Paper IV

Family stress indices

Five questions regarding parental discord were answered on the two-year questionnaire, as well as six questions regarding parenting problems and child temperament. All of these questions were answered on a Likert scale. The exact translated wording can be seen in Figure 17. For analysis, we recoded all answers as a value between 0 and 1, giving all questions equal weight. The recoded values were then added up to an index value. Index values above the 90th percentile were considered to be positive in the analysis. Separate indices were calculated for

answers given by the mother, answers given by the father and a joint variable with index values from both parents combined.

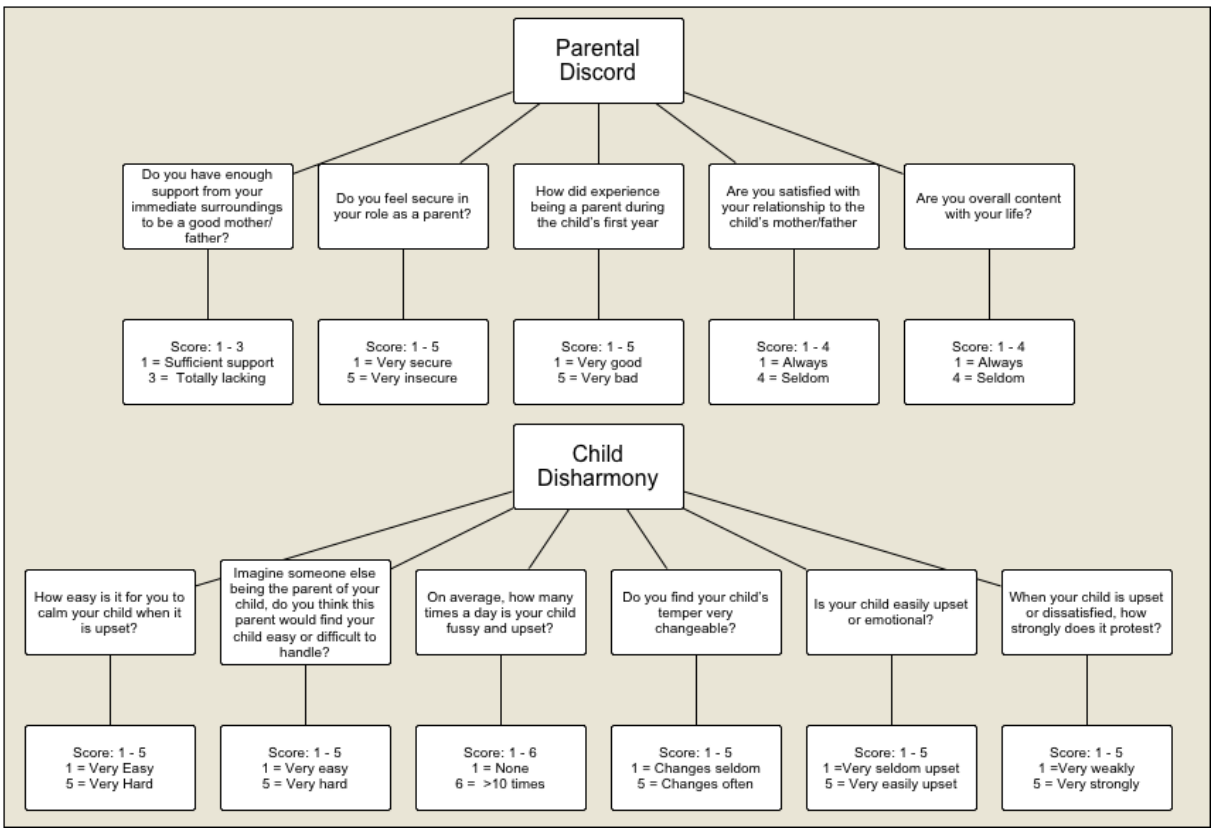

Figure 17. Questions and possible answers included in the parental discord and Child disharmony indexes

Tau-equivalent reliability was calculated using Cronbach's α, showing acceptable internal consistency for maternal and paternal answers on parental discord when analyzed separately (Cronbach's α coefficient of 0.65 for mothers and 0.59 for fathers) and good internal consistency for the joint parental index (Cronbach's α coefficient of 0.76). For the Parenting Stress index, internal consistency was good regarding the separate maternal and paternal answers (Cronbach's α coefficient of 0.78 for mothers and 0.76 for fathers), and consistency was excellent for the joint parental index (Cronbach's α coefficient of 0.86). The distribution of index values for both indices can be seen in Figure 18.

Statistical analysis

To assess potential confounders in the model, a univariate Cox proportional hazards model was used. Confounders with a significance value of $p < 0.2$ were included in a multivariate Cox proportional hazards model. The included confounders included maternal insulin-dependent diabetes, paternal insulin-dependent diabetes, IA-2A cord blood positivity[325], mother born in Sweden and father born in Sweden. The multivariate analysis was stratified for HLA risk group (DQ2/8, DQ8/8 or DQ8/X, DQ2/2 or DQ2/X and DQX/X, (X is neither 8 nor

2)). To further examine the possible effects of stress, a separate analysis was performed for the highest-risk group: HLA DQ2/DQ8. In this multivariate model, maternal insulin-dependent diabetes, paternal insulin-dependent diabetes, IA-2A cord blood positivity and paternal education level were used as covariates. In the sub group analysis, the SLE "Own divorce or separation" failed to meet the requirements of the statistical model and was not included in the results.

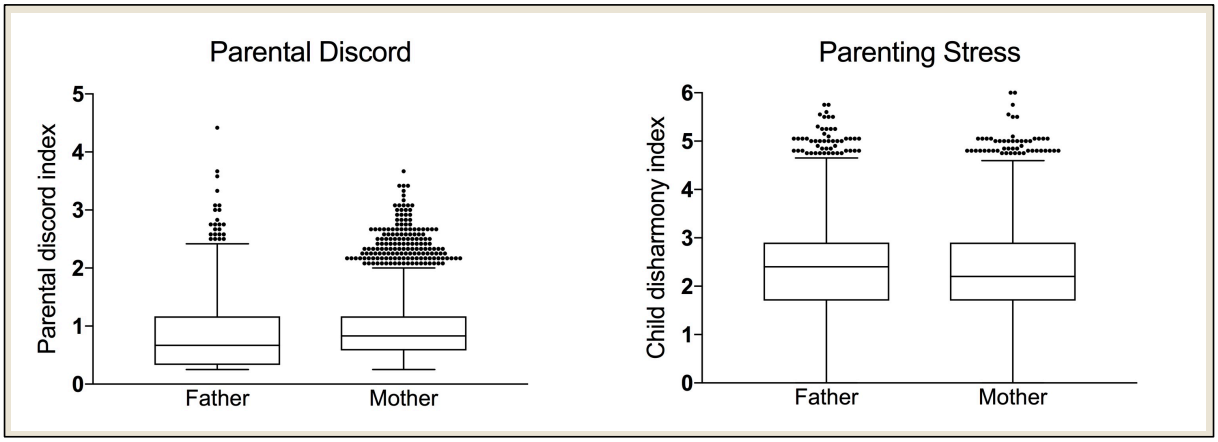

Figure 18. Tukey box-plot of answers to the parental discord and parenting stress indices.

Censoring for cases that had not been diagnosed with T1D was set at April 20th, 2016. Missing data in questionnaires were handled in a pairwise manner. Statistical analysis was performed using SPSS version 24 (IBM Corp, Armonk, NY, USA) and GraphPad Prism 7 (GraphPad Software, La Jolla, California, USA).

Paper III

Study population and data acquisition

For the analysis in paper III, the data received at the TEDDY data coordinating center as of 31st of December 2014 were used. At this time, 8,876 children were enrolled in the study. For this analysis, only children with medication data regarding for the first two years of life, first child status available and islet autoantibody status were included. After exclusion due to missing data regarding medication use, first child status and participants lacking full SNP data, 6,894 participants remained for the analysis. Study inclusion can be seen in Figure 19. At study visits, every three months, interviewers recorded all medication, with the name of the drug, the start date and the duration since the last study visit. Data regarding infections were documented by the parents in the "TEDDY Book" and for every illness episode, parents answered "yes" or "no" regarding the presence of

fever. All parents received written guidance that "yes" should only be selected if the body temperature was ≧38°C/101° F.

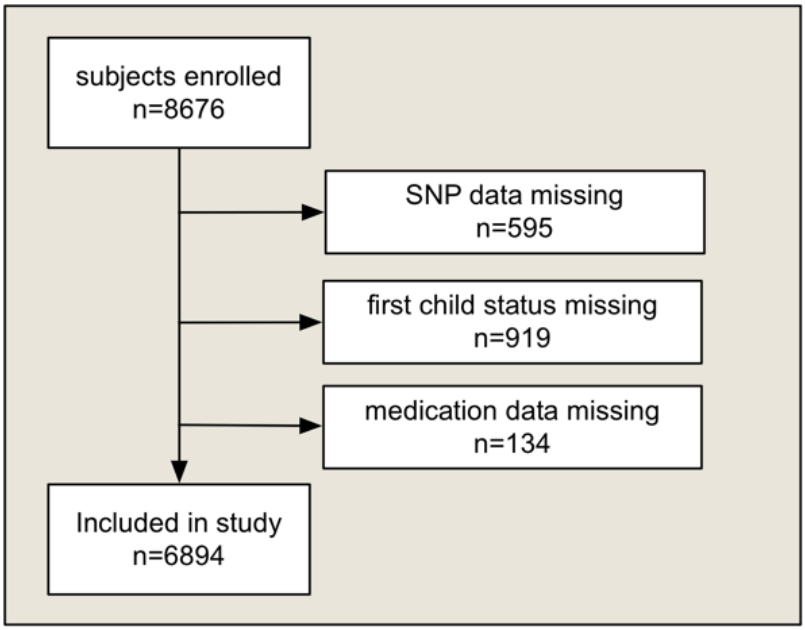

Figure 19. Excluded subjects in Paper III.
A total of 1648 subjects were excluded due to missing data leaving a total of 6894 subjects included in the study.

Approximately 18 months into the study, these choices were expanded to "Yes – measured," "Yes – not measured," and "No". All illness was recorded as ICD-10 codes, start of episode and duration. Infection was defined as an ICD-10 code defined as infection by the study clinical implementation and infectious agents committee. The method for reporting and categorizing illness in TEDDY has been previously reported elsewhere[326]. Medication was recorded by brand name and categorized according to active ingredient. For analysis, all medication including that ingredient was included. For further analysis, each medication was also categorized as either acetaminophen(APAP) or non-steroidal anti-inflammatory (NSAID). Each use of an analgesic antipyretic (ANAP) was defined as an episode and was defined as associated with infection and/or fever.

Statistical analysis

The impact of ANAP use in the first 90, 180, and 365 days of life, as well as the first 2.5 years of life, on IA at age three and six years was assessed using a Cox proportional hazards model in which country was used as a stratification variable. The number of infections early in life was added as a time-dependent covariate[327]. As additional covariates, first-degree relative[328], HLA[159], gender, ever breastfed[329,330], probiotic use before 3 months of age[233] and eight single nucleotide polymorphisms previously identified in the TEDDY study were included[331]. Cumulative use of ANAPs during the child´s first 2.5 years was used as the primary covariate of interest. In cases with missing data regarding the duration of

use, that subject was excluded from the analysis for that specific analgesic. To better fit the linear statistical model, log transformed data were used for the analysis of the total duration of ANAP use per year. A logistic regression model was used to analyze subject incidence, with country and first child status as independent variables. Episodes were categorized as either with concurrent fever (yes/no) or infection (yes/no), both fever and infection (yes/no) or neither fever nor infection (yes/no). Using an ignorable working index, a generalized estimating equation (GEE) was used for analysis, with country and first child status as independent variables. An ignorable working matrix was assumed for the GEE analysis, with the empirical sandwich estimate used for the standard errors. For pairwise comparisons, in both binary and continuous analyses, we corrected for multiplicity of comparisons using the Bonferroni-Holm procedure.

Statistical analyses were performed using the Statistical Analysis System software, version 9.3 (SAS Institute Inc., Cary, NC, USA). Figures were created using GraphPad Prism 7 for Mac (GraphPad Software, La Jolla California, USA).

Paper IV

Study population and data acquisition

In paper IV, we aimed to analyze the status at diagnosis and for the two first years after diagnosis, focusing on whether the children followed in the DiPiS study differed from children in the general population. Before the age of two years, at which follow-up began, 14 children had been diagnosed with T1D. These children were excluded from the analysis. In the DiPiS cohort, 129 children had been diagnosed with T1D as of July 31st, 2013. Risk information was only offered to children whose parents consented to follow-up at age two. Out of these children, 4,340 children consented to follow-up, and 82 of those children had been in intense follow-up every three months. The remaining 4,258 children participated in yearly follow-up (Figure 20.). For this study, all children who had received information about T1D risk and participated in follow-up at any time formed the Follow-up group. The Non-follow-up group consisted of children who chose not to participate in the 2-month questionnaire, who were not offered follow-up or who chose not to participate in follow-up. These children had not received information regarding T1D risk. At the time of analysis, 32 participants had not yet reached 24 months post-diagnosis, including 14 in the follow-up group and 18 in the non-follow-up group. The number of participants available for analysis can be seen in Table 4. HLA DQ genotyping and autoantibody analysis were performed as previously described in the methods section of this thesis. Prevalence data regarding diabetes diagnosis were reported from pediatricians at the pediatric

clinics in Region Skåne at the time of diagnosis. Additional prevalence data were retrieved from the Better Diabetes Diagnosis study (BDD)[134] and the Skåne study.

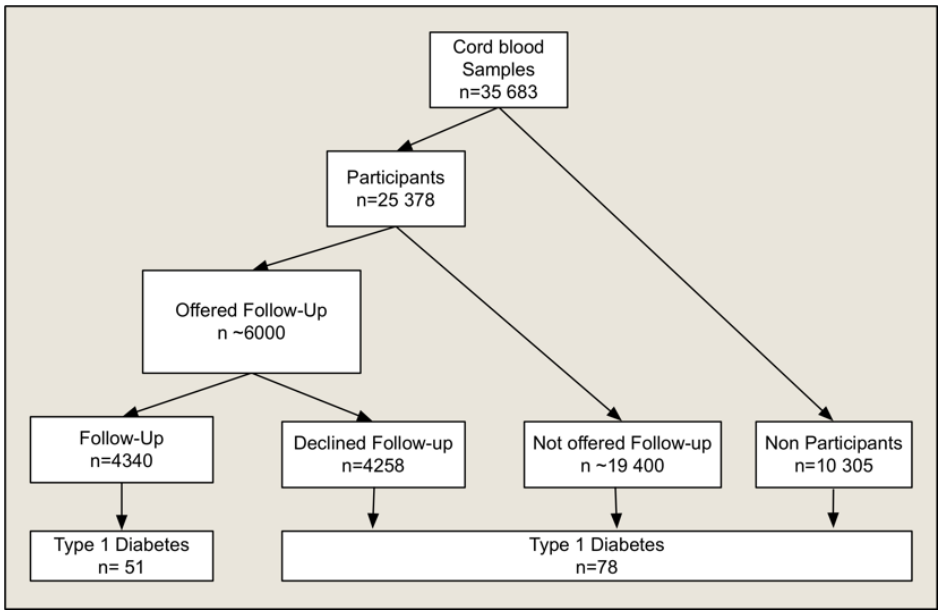

Figure 20. Flow diagram of study participants in Paper I

Diagnosis data and data regarding follow-up (HbA1c and insulin doses) were retrieved from electronic patient medical records (Melior, Siemens AG, Berlin, Germany). Insulin doses were analyzed as the total daily dose (TDD) of insulin per kg bodyweight. A +/- one-month window was allowed for follow-up data to maximize data retrieval. DKA was defined as blood pH <7.3, with severe DKA defined as pH <7.1.

Table 4. Follow-up data available for analysis after diagnosis

Group	Diagnosis	3 months	6 months	12 months	24 months
Follow-up	**51**	**45**	**45**	**43**	**33**
No Follow-up	**77**	**74**	**74**	**70**	**56**

Statistical methods

Statistical analysis was performed via group-wise comparisons between the follow-up and non-follow-up groups. Categorical variables were tested using Pearson's χ^2 or Fischer´s exact test, where applicable. The Mann-Whitney U test was used for continuous variables. Spearman´s rho was used for correlation analysis between Hba1c and insulin doses during follow-up. HbA1c was recorded and analyzed as IFCC (mmol/mol) and converted to NGSP (%) for presentation purposes[332]. Corrections for multiple comparisons were performed using the Bonferroni method, where applicable. SPSS 21 was used for statistical analysis (SPSS Inc., Chicago, IL, USA).

Paper V

Study population and data acquisition

According to the original study plan, 50 participants were recruited to the study. The participants were randomized to either two doses of 20 µg of GAD-alum or placebo as a subcutaneous injection, with one month between injections. All participants and study personnel were blinded during both treatment and follow-up. After the administration of the study drug or placebo, the participants were followed for five years or until a diabetes diagnosis. Glucose tolerance tests were performed every six months, alternating between OGTT and IvGTT. At all visits, laboratory samples were collected, including islet autoantibodies (GADA, IAA, IA-2A, ZnT8 RA/WA/QA), hematology, blood chemistry, HbA1c, random plasma glucose and random or fasting C-peptide (depending on visit). Annual screening for celiac and thyroid autoimmunity was performed.

Information from the study visits was entered into a clinical research form (CRF) and archived both electronically and on paper. Adverse events were graded as mild, moderate or severe. All alterations in health status were recorded as adverse events.

Glucose tolerance tests

OGTT was performed after overnight fasting. An oral dose of 1.75 g of glucose/kg body weight of Nutrical (N.V. Nutrical Zoetermeer, Holland) was given. At 0, 30, 60, 90 and 120 minutes after ingesting the venous plasma glucose, serum C-peptide and serum insulin were measured.

IvGTT was performed after overnight fasting. An intravenous dose of 500 mg/kg body weight of glucose was injected within 3 minutes as a 300 mg/ml solution. At -10, 0, 1, 3, 5, 7, 10, 30, 50, 70 and 90 minutes after injection, a venous sample was collected for the analysis of plasma glucose, serum C-peptide, and serum insulin. Glucose values were plotted semi-logarithmically against time. The disappearance rate of glucose was estimated on the near-straight line in order to calculate the K-value[333].

Statistical analysis

All 50 children included in the study were included in the final statistical analysis. Unblinding was performed after the database was locked and assigned to the intent to treat, full, per protocol and safety populations. All statistical analysis and grouping of participants were performed according to the statistical analytical plan outlined in the original protocol. One child was excluded from the per protocol analysis due to diabetes values at the baseline visit.

For analysis, non-normally distributed numeric variables were log transformed, then back-transformed for presentation. A K-test or Wilcoxon´s signed rank test

was used to compare baseline characteristics for continuous variables. Pearson's χ^2 or Fischer´s exact test was used for categorical variables. Using log-rank $\chi 2$ statistics, Kaplan-Meier life tables were compared. A univariate Cox proportional hazards model was used to compare risk between groups. A multivariate Cox model was used to account for possible covariates affecting the results. This model included sex (girl/boy), treatment with GAD-Alum (yes/no), age at randomization, first-degree relative (yes/no), impaired glucose tolerance at baseline (yes/no), baseline FPIR, baseline C-peptide area under the curve (AUC) and autoantibody stratum (2 autoantibodies/3-6 autoantibodies). The assumptions of the Cox proportional hazards model were confirmed for all variables.

A mixed-models repeated measures approach was used to analyze longitudinal data on secondary end-points during follow-up. Subject effects were treated as random effects, and time and the variable of interest were treated as fixed effects. The validity of mixed models was examined using goodness of fit statistics (log likelihood deviance, AIC, BIC). No adjustments were made for multiplicity of comparison, except when part of the multivariate analysis. All tests of significance were two-tailed, and the significance level was set at 0.05.

Statistical analysis was performed using SAS 9.3 (SAS Institute, Inc., Cary, NC, USA).

Ethical approval

Ethical approval for papers I, II and IV was granted by the ethics review board in Lund, Sweden via approvals for the DiPiS study.

Paper III received ethical approval from the Colorado Multiple Institutional Review Board, the Augusta University Institutional Review Board, the University of Florida Health Center Institutional Review Board, the Western Institutional Review Board, the Ethics Committee of the Hospital District of Southwest Finland, the Bayerischen Landesärztekammer (Bavarian Medical Association) Ethics Committee, and the Lund University Committee for Continuing Ethical Review. The study is also overseen by an external advisory board formed by the National Institutes of Health. The data that support the findings of paper III will be available from the NIDDK repository (http://niddkrepository.org) approximately 6 months after the manuscript has appeared in print.

Approval for paper V was granted by the ethics review board in Lund, Sweden, as well as by the medical product agency (MPA) in Sweden.

Results

Do umbilical cord blood islet autoantibodies increase the risk of T1D (Paper I)?

Baseline characteristics of the study

Umbilical cord blood samples from 35,683 children were collected at birth as part of the DiPiS screening. Baseline data were collected from both data collected at birth and from the two-month questionnaire answered by the parents of 25,392 children. Of the parents who responded to the two-month questionnaire, 190 mothers reported insulin-treated diabetes and 790 mothers reported gestational diabetes. Paternal insulin-treated diabetes was reported for 285 children. At the time of analysis (December 31st, 2013), 151 children from the originally screened cohort had been diagnosed with T1D at a median age of 5.8 years (range: 0.8-12.2 years).

How many children are islet autoantibody-positive at birth?

Islet autoantibodies against one or more islet autoantibodies were found in umbilical cord blood from 757 children (2.1%). GADA were found in 295 children (0.8%), IA-2A were found in 78 children (0.2%) and IAA were found in 509 children (1.4%). Of the IAA-positive children, 105 (21%) had mothers with insulin-treated diabetes, 214 (42%) had mothers who were not treated with insulin and 190 (37%) were missing data regarding maternal insulin use. The distribution of single-, double- and triple-positive samples can be seen in figure 21.

Is the T1D risk higher for children with cord blood islet autoantibodies?

All probable covariates were initially analyzed in a univariate model. In this analysis, IA-2A (HR 13.5; 95%CI 5.02, 36.6; p <0.001) and GADA (HR 3.44; 95% CI 1.27, 9.29; p=0.015) but not IAA (HR 2.41; 95% CI 0.98, 5.88; p=0.053) were predictors of T1D risk.

When using a multivariate model, with stratification for HLA risk group and with maternal insulin-treated diabetes, paternal insulin-treated diabetes, IA-2A, GADA and IAA included as covariates, only IA-2A showed evidence of association with T1D risk (HR 7.73; 95% CI 1.94, 30.7; p <0.001). This result

persisted in an expanded model, in which possible confounders (relative birth weight, infections during pregnancy, maternal age, gestational length and maternal age) were included (HR 6.88; 95% CI 1.46, 32.4; p <0.001). GADA positivity was not significant after adjusting for confounders in the multivariate model (HR 1.5; 95% CI 0.5, 4.8; p=0.82)

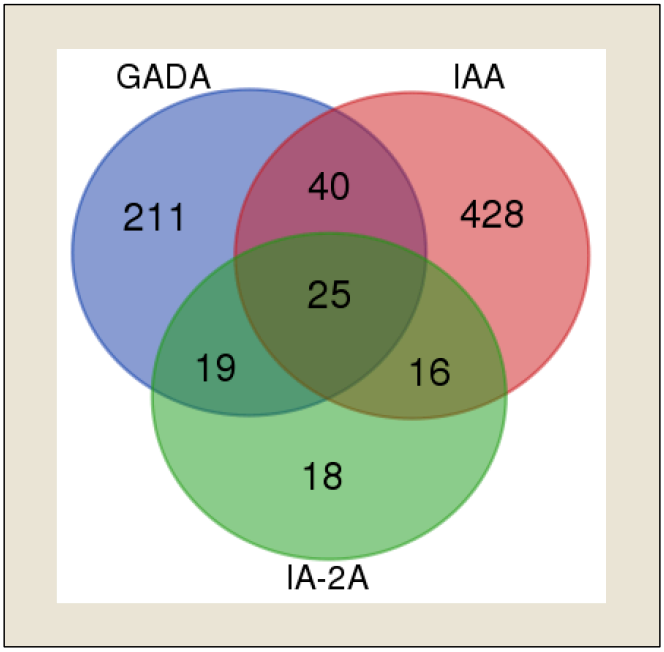

Figure 21. Distribution of islet autoantibodies in cord blood.

To analyze the possible correlation between maternal diabetes status, IA-2A positivity and T1D diagnosis, contingency tables were used. Among children of mothers with insulin-treated diabetes, the presence of IA-2A in the cord blood was associated with development of T1D in the child (p=0.037). However, among children with IA-2A in cord blood, maternal diabetes was not associated with the development of T1D in the child (p=1). The univariate and full multivariate Cox results are presented in Table 5.

Table 5. Association between selected factors and the diagnosis of T1D.

	Univariate HR (95% CI)	p value	Multivariate HR (95% CI)	p-value
IA-2A-positive	13.5 (5.02-36.6)	<0.001	6.88 (1.46-32.4)	0.015
IAA-positive	2.41 (0.98-5.88)	0.053	0.65 (0.14-3.08)	0.58
GADA-positive	3.44 (1.27-9.29)	0.015	1.11 (0.27-4.50)	0.88

What are the results regarding other risk factors?

The risk conferred by the HLA risk group (HLA DQ2/8, DQ8/8 or DQ8/X, DQ2/2 or DQ2/X, DQX/X) was similar to the risk values reported in earlier studies (Table

6.). Having a father with insulin-treated diabetes was associated with an increased risk of T1D (HR 3.4; 95% CI 1.6, 7.0; p <0.001). Having a mother with insulin-treated diabetes was significant in the univariate analysis (HR 3.5; 95% CI 1.3, 9.5; p=0.014) but failed to reach significance in the multivariate analysis (HR 1.38; 95% CI 0.24; 7.84; p=0.71).

Table 6. Association between HLA risk group and T1D risk.

	Univariate Analysis HR (95% CI)	p value	Multivariate Analysis HR (95% CI)	p-value
HLA risk-group				
-DQ 2/8	**18.2 (13.1-25-3)**	**<0.001**	**42.4 (23.3-77.2)**	**<0.001**
-DQ 8/8 or 8/X	**3.48 (2.42-5.01)**	**<0.001**	**10.9 (5.74-20.7)**	**<0.001**
-DQ 2/2 or 2/X	**2.02 (1.30-3.16)**	**0.002**	**8.51 (4.20-17.2)**	**<0.001**
-DQ X/X (ref)	**ref**	**ref**	**ref**	**ref**

Are stress and severe life events in early life a risk factor for T1D (Paper II)?

Baseline characteristics of the study

Responses to the two-month questionnaire were received from the parents of 23,187 children, and responses to the two-year questionnaire were received from the parents of 3,861 children. The mean age at T1D diagnosis in this cohort was 8.19 years (SD 3.8) (Table 7.).

Do early severe life events increase the risk of T1D?

In the total cohort, using a univariate cox regression model, only having experienced violence during or after pregnancy was associated with an increased risk of T1D (HR 4.42; 95% CI 1.41, 13.88; p=0.011). However, in multivariate analysis stratified for the HLA risk group, having experienced any severe life event after birth was associated with an increased risk of T1D (HR 1.66; 95% CI 1.02, 2.70; p=0.043) as was conflict with spouse (HR 2.28; 95% CI 1.19, 4.36; p=0.013) and having been the victim of violence (HR 4.52; 95% CI 1.29, 15.8; p=0.018). For the high-risk HLA DQ2/8 sub cohort, univariate analyses show an increased risk of T1D among children of parents who experienced any of the severe life events after pregnancy (HR 2.15; 95% CI 1.09, 4.27; p=0.028) (Figure 22.), as well as parents who had been the victim of violence (HR 7.42; 95% CI 1.81, 30.5; p=0.005).

Table 7. Baseline characteristics of respondents to the 2-month and 2-year DiPiS questionnaires.

		2-month Questionnaire	2-year Questionnaire
Number of questionnaires	**n**	**23187**	**3861**
Maternal age at birth	**Mean (IQR)**	**30,8 (6,0)**	**31.4 (6.0)**
Male child	**n (%)**	**11589 (51.6)**	**1899 (52.2)**
Gestational age	**Median (IQR)**	**39,3 (2,0)**	**39.1 (2.0)**
Maternal IDDM	**n (%)**	**163 (0.7)**	**117 (3.1)**
Paternal IDDM	**n (%)**	**227 (1.0)**	**125 (3.3)**
HLA risk group			
DQ2/DQ8	**n (%)**	**865 (3.7)**	**431 (11.4)**
DQ8/8 or 8/X	**n (%)**	**2163 (9.3)**	**1140 (30.1)**
DQ2/2 or 2/X	**n (%)**	**1937 (8.4)**	**1016 (26.8)**
DQ X/X	**n (%)**	**18190 (78.6)**	**1197 (31.6)**
Maternal education			
Nine-year compulsory school	**n (%)**	**1412 (6.1)**	**196 (5.2)**
Senior high school	**n (%)**	**11611 (50.4)**	**1812 (48.1)**
College/University	**n (%)**	**10028 (43.5)**	**1761 (46.5)**
Paternal education			
Nine-year compulsory school	**n (%)**	**2079 (10.0)**	**336 (9.6)**
Senior high school	**n (%)**	**11411 (49.2)**	**1885 (53.7)**
College/University	**n (%)**	**7379 (35.4)**	**1287 (36.7)**
Mother born in Sweden	**n (%)**	**20419 (89.2)**	**3472 (93.0)**
Father born in Sweden	**n (%)**	**18553 (88.8)**	**3434 (92.1)**

In the multivariate model that included HLA risk-group stratification, having experienced any severe life event after pregnancy was still significantly correlated with T1D risk (HR 2.21; 95% CI 1.08, 4.51; p=0.030), as was the father being unemployed (HR 3.50; 95% CI 1.09, 11.3; p=0.036) and conflict with spouse (HR 3.12; 95% CI 1.22, 7.99; p=0.018).

Are self-reported parenting stress and parental discord correlated with increased T1D risk?

Using the parental discord index in the total cohort, answers above the 90th percentile given by the father were associated with an increased T1D hazard ratio in only the univariate analysis (HR 1.98; 95% CI 1.04, 3.78; p=0.038). No other significant hazards for the parental discord index were found in multivariate analysis, either when analyzing the parental answers separately or when analyzing the parental answers as a joint variable (mother p=0.41; father p=0.27; joint p=0.28). Index scores above the 90th percentile for the parenting stress index were not correlated with an increased T1D risk for the joint answers from both parents or for the answers from the mother or father separately (p=0.532; p=0.772; p=0.335). For the high-risk HLA DQ2/8 sub-cohort, parental discord, as reported

by the father and the joint parental answers, correlated with an increased T1D risk (HR 2.68; 95% CI 1.02, 7.08; p=0.047 and HR 2.87; 95% CI 1.17, 7.10; p=0.022, respectively) in the univariate analysis.

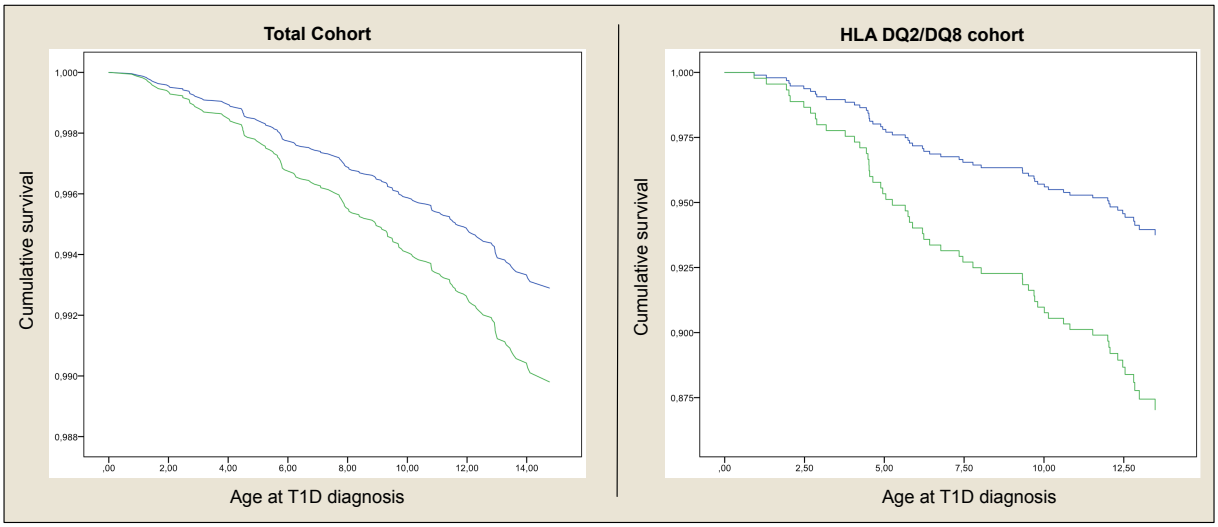

Figure 22. Kaplan Meier curve for severe life events after pregnancy. Separate curves are resented for the total study cohort and the HLA DQ2/DQ8 sub-cohort. *Green line: Yes; Blue line: No*.

Reported parental discord from the mother in the univariate analysis (p=0.054) and the indices from both parents in the multivariate analysis did not correlate with the T1D risk (mother p=0.17; father p=0.11; combined p=0.082). No correlation with T1D risk was found for the parental stress index in the univariate (mother p=0.68; father p=0.41; combined p=0.90) or multivariate analysis (mother p=0.80; father p=0.68; combined p=0.86).

Is the use of analgesic antipyretics in early life a risk factor for islet autoimmunity (Paper III)?

Baseline characteristics of the study

The study cohort consisted of 8,542 children, 51% male and 11% first-degree relatives, who were enrolled in the TEDDY study. The country distribution can be seen in Table 9. In general, the use of APAP and NSAIDs before the age of 2.5 years was very common in the studied cohort. Parents reported that 87.8% of children had used APAP and 45.4% had used NSAIDs at any time. The mean number of treatment episodes was reported to be 3.6 (SD 2.1), with a mean duration of treatment per year of 8.5 days (SD 10.8) (Figure 23.).

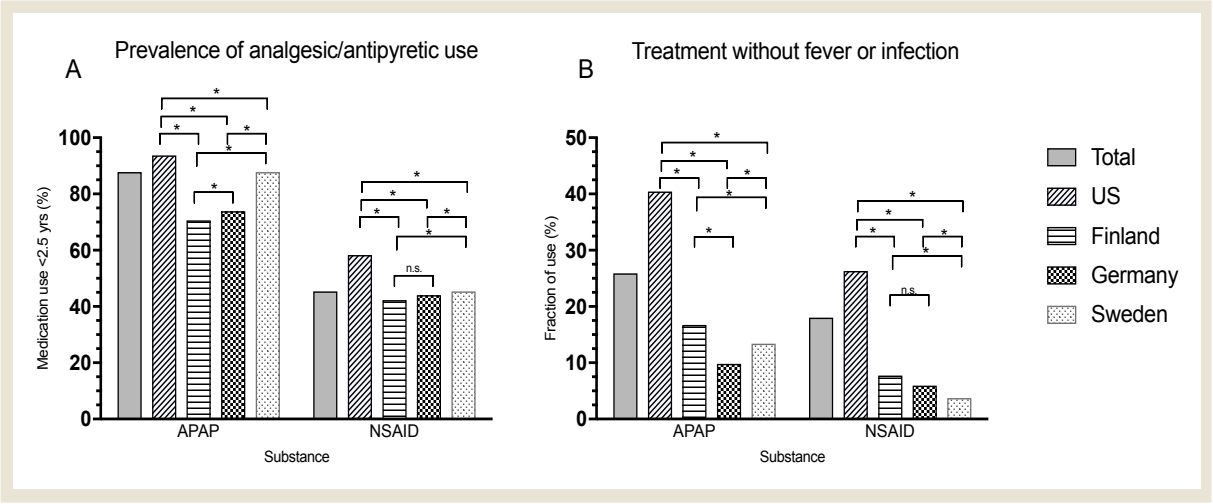

FIGURE 23. Use of ANAP below 2.5 years of age. A: Prevalence of analgesic/antipyretic use. B: Prevalence of analgesic/antipyretic use in the absence of fever or infection. All significances were corrected for multiple comparisons using the Holm procedure. *: p<0.05, n.s.: non-significant

Analgesic antipyretics and the risk of islet autoimmunity

Analysis was performed for the cumulative use of APAP or NSAIDs, with or without concurrent fever, as well as for a joint, cumulative variable of total ANAP use with or without fever. Only cumulative APAP use with concurrent fever or infection before age 2.5 years was associated with a significant hazard. However, the increased risk was only significant for IA at three years of age (HR 1.05; 95% CI 1.01, 1.09; p=0.024) but not at six years of age (HR 1.03; 95% CI 0.99, 1.08; p=0.189). Further analysis of the time of exposure, found a significant hazard correlated with T1D risk for APAP exposure with fever or infection before one year of age (HR 1.06; 95% CI 1.00, 1.12; p=0.011). No other exposures to APAP or NSAIDs, with or without fever/infection, before 90, 180 or 365 days of life were correlated with T1D risk (Table 8.).

Table 8. Hazard ratios for seroconversion to persistent IA at 3 and 6 years for analgesic variables of interest.

Analgesic Variable	Exposed subjects n (%)	3-Year Analysis 398 antibody$^+$ subjects HR (95% CI) p-value	6-Year Analysis 511 antibody$^+$ subjects HR (95% CI) p-value
Acetaminophen, any exposure	**7496 (87.8%)**	**1.01 (0.97, 1.05) 0.603**	**1.01 (0.98, 1.05) 0.576**
Acetaminophen with fever + infection	**5179 (60.6%)**	**1.05 (1.01, 1.09) 0.022**	**1.03 (0.99, 1.08) 0.189**
Acetaminophen without fever or infection	**5941 (69.6%)**	**1.02 (0.98, 1.06) 0.346**	**1.01 (0.96, 1.05) 0.769**
NSAID, any exposure	**3874 (45.4%)**	**1.01 (0.97, 1.05) 0.753**	**1.01 (0.97, 1.04) 0.763**
NSAID with fever + infection	**2652 (31.0%)**	**1.01 (0.97, 1.05) 0.673**	**1.01 (0.98, 1.05) 0.459**
NSAID without fever or infection	**1955 (22.9%)**	**1.00 (0.96, 1.05) 0.856**	**1.01 (0.97, 1.05) 0.623**
Any analgesic, any exposure	**7744 (91%)**	**1.02 (0.99, 1.05) 0.130**	**1.02 (0.99, 1.04) 0.267**
Any analgesic with fever + infection	**5699 (67%)**	**1.06 (0.97, 1.15) 0.219**	**1.02 (0.94, 1.10) 0.667**

Acetaminophen use in young children in the TEDDY cohort

The highest prevalence of APAP use was reported among Swedish parents (94.5%), with the US being a close second (93.7%), and Finland (73.9%) and Germany (70.1%) reporting significantly lower prevalence rates. The significance of the differences between the sites can be seen in figure 23.

The highest number of treatment episodes was reported for the US children, followed by the Swedish, Finnish and German children. The number of treatment episodes is summarized in Table 9. APAP was used more often during the child's first 2.5 years by children born as the first child in the family in comparison to children with older siblings (OR 1.26; 95% CI 1.07, 1.49; p=0.007). First-born children had a higher number of treatment episodes (difference in least square means 0.15; 95% CI 0.05, 9.24; p=0.003), but no difference could be seen in the number of days treated (difference in least square means 0.11; 95% CI -0.38, 0.60; p=0.11)

Table 9. Analgesic/Antipyretic by country and for the total cohort.

		US	Finland	Germany	Sweden	Total Cohort
Subjects	**n**	**3662**	**1795**	**581**	**2504**	**8542**
Treatment episodes (n)						
Acetaminophen	**mean (SD)**	**4.0 (2.3)**	**2.7 (1.9)**	**2.5(1.6)**	**3.6 (1.7)**	**3.6 (2.1)**
NSAID	**mean (SD)**	**2.1 (1.4)**	**1.6 (1.2)**	**1.6 (1.1)**	**1.6 (1.2)**	**1.9 (1.3)**
Total treatment (days)						
Acetaminophen	**mean (SD)**	**9.8 (13.5)**	**6.8 (6.4)**	**4.7 (3.7)**	**8.4 (8.6)**	**8.5 (10.8)**
NSAID	**mean (SD)**	**6.8 (11.2)**	**5.3 (9.3)**	**5.2 (17.2)**	**4.9 (4.8)**	**6.0 (10.5)**

NSAID use in young children in the TEDDY cohort

The US hade the highest prevalence of NSAID use before age 2.5 years (53.8%). No difference in use could be seen between Germany (44.1%) and Finland (42.3). Sweden had the lowest prevalence of NSAID use (29.0%). The significance of country-wise comparisons of use at the different sites can be seen in table 11.

The highest number of treatment episodes was reported for children from the US, followed by Swedish, Finnish and German children. In addition, regarding the total duration of treatment, the US reported the highest number of days, followed by Finland, Germany and Sweden, with no significant difference observed between the countries (Table 10.). First-born children had a lower prevalence of NSAID use during their first 2.5 years of life than children with older siblings (OR 0.86; 95% CI 0.78, 0.95; p=0.002). First-born children were also treated fewer times (difference in least square means -0.14; 95% CI -0.22, -0.05; p=0.001). No

differences could be seen regarding the number of days treated for first-born children (difference in least square means -0.22; 95% CI -0.92, 0.48; p=0.143).

Table 10. Statistical significance of country differences regarding ANAP use.

	U-F	U-G	U-S	F-G	F-S	G-S
Prevalence						
Acetaminophen	<0.001	<0.001	<0.001	0.035	<0.001	<0.001
NSAID	<0.001	<0.001	<0.001	0.177	<0.001	<0.001
Number of treatment episodes						
Acetaminophen	<0.001	<0.001	<0.001	0.014	<0.001	<0.001
NSAID	0.001	<0.001	<0.001	0.485	0.519	0.810
Total duration of treatment						
Acetaminophen	<0.001	<0.001	0.739	<0.001	<0.001	<0.001
NSAID	<0.001	<0.001	<0.001	0.181	0.674	0.105

U=the US, G=Germany, F=Finland, S=Sweden.

In what context are TEDDY children given analgesic antipyretics?

In the total cohort, the majority of both APAP (74.1%) and NSAID (82.0%) doses were given with concurrent fever, infection or both, with 43.8% of APAP doses and 51.0% of NSAID doses given in the context of both infection and fever. The US parents reported significantly higher use in the absence of both fever and infection for both APAP (40.4%) and NSAIDs (26.3%) in comparison to the other three countries ($p < 0.001$).

The use of APAP in conjunction with fever was most common among German and Swedish children (68.5% and 63.2%, respectively), followed by Finland (57.9%) and the US (23.5%). All differences between countries were significant, with $p < 0.001$, except for the differences between Germany and Sweden, for which p=0.003.

The US reported the highest proportion of NSAID doses given in the absence of both fever and infection (26.3%), followed by Finland (7.7%), Germany (5.9%) and Sweden (3.7%). All country differences were statistically significant, with $p < 0.001$, except for the differences between Finland and Sweden (p=0.006) and Germany and Sweden (p=0.01) (Figure 23.).

Does participation in longitudinal follow-up affect peri-diagnosis morbidity and short-term glycemic control (Paper IV)?

Baseline characteristics of the study

The cohort of paper IV consisted of a total of 129 children, including 67 girls (52%), with a mean age at diabetes diagnosis of 6.6 years (SD 2.7 years). The children in the follow-up and non-follow-up groups in paper IV did not differ with respect to gender (p=0.38) or age at diabetes diagnosis (p=0.45). Two children were islet autoantibody-negative at the time of diagnosis. However, both of those cases were diagnosed with T1D, with one case being antibody-positive in confirming samples and one case presenting with clinically typical T1D.

Does the status of the children at the time of T1D diagnosis differ for children enrolled in DiPiS follow-up compared to controls?

At diagnosis, the children in the follow-up group had a lower frequency of diabetic symptoms, a higher percentage of children diagnosed without any symptoms and a lower frequency of DKA. No children that had been in intense follow-up, one child who had previously been in follow-up and three children who had not been in follow-up and had risk information presented with severe DKA. Complete results can be seen in table 11.

Table 11. Symptoms at diagnosis. Comparison between the Follow-up group and the Non-follow-up group.

	n	Follow-up Prevalence (%)	No Follow-up Prevalence (%)	OR	95% CI	p	p_{corr}*
Symptom, any	129	84	97	0.14	0.03; 0.70	0.014	0.028
Polydipsia	129	80	96	0.16	0.04; 0.63	0.006	0.018
Polyuria	129	76	96	0.13	0.04; 0.49	0.001	0.003
Weight loss	122	46	65	0.45	0.22; 0.95	0.034	0.10
DKA	129	2	18	0.09	0.01; 0.72	0.005	0.01
DKA, Severe	129	2	5	0.5	0.05; 4.94	1.0	1.0

DKA, Diabetic Ketoacidosis (pH <7.3); Severe DKA, Diabetic ketoacidosis (pH<7.1);
** Bonferroni-corrected p-value; Reprinted with permission from Wiley*[334]

Does prospective follow-up influence glycemic control during the first two years after diagnosis?

At diagnosis, the follow-up group had significantly lower median levels of Hba1c than the non-follow-up group (77 mmol/mol (IQR 64-91) and 87 mmol/mol (IQR 75-99), respectively; p=0.006; p_{corr}=0.03). During follow-up after diagnosis, significant differences could be seen at both 12 months post-diagnosis (53

mmol/mol (IQR 48-57) and 57 mmol/mol (IQR 51-65), respectively; p=0.009; p_{corr}=0.045) and 24 months post-diagnosis (53 mmol/mol (IQR 48-61) and 62 mmol/mol (IQR 54-69), respectively ($p < 0.009$; $p_{corr} < 0.001$)). No significant differences in HbA1c could be seen at three and six months post-diagnosis (p=0.12; p=0.34) (Figure 24.).

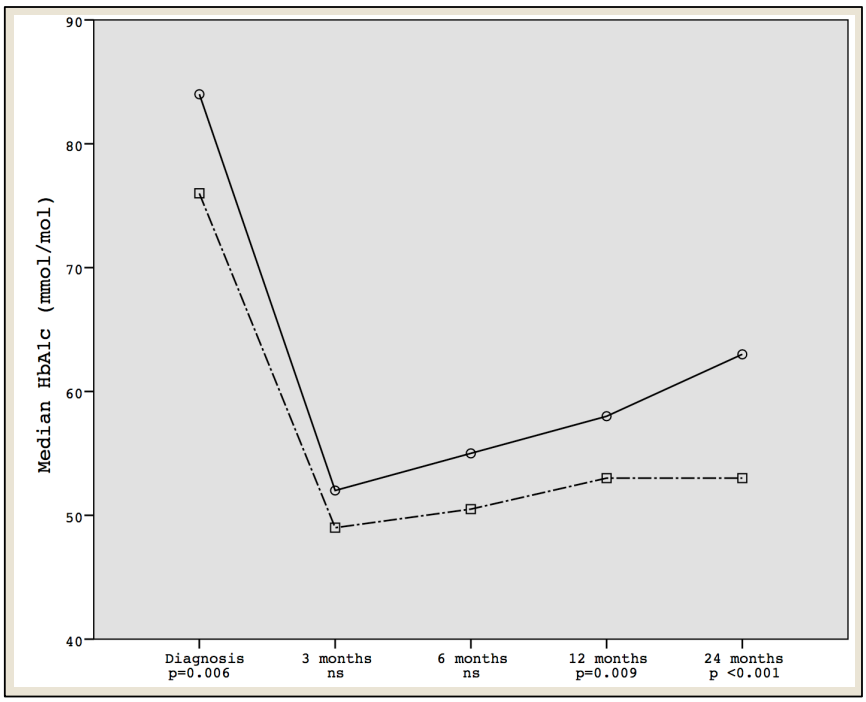

Figure 24. HbA1c during the 2 years after follow-up. Reprinted with permission from Wiley[334].

Can the differences in glycemic control be explained by insulin dosage or remission?

Only at three months after diabetes diagnosis could a significant difference in insulin dose be seen when calculated as the total daily dose (TDD), with the follow-up group reporting a lower median dose of insulin (p=0.026). However, after adjusting for multiple comparisons, this difference was not significant.

Partial remission, which was defined as TDD <0.5 U/kg/day, was not significantly more common in the follow-up group than the non-follow-up group. In an unpublished analysis, partial remission was also analyzed using insulin dose-adjusted HbA1c (IDAA1C=NGSP % + 4 x TDD)[335]. No significant differences could be seen at any time point (Table 12.).

Table 12. Insulin dose-adjusted HbA1c during the two years after diagnosis.

	n	Follow-Up Median (IQR)	No Follow-Up Median (IQR)	p
IDAA1C 3 months	122	8.5 (7.7-.9)	8.8 (8.1-9.9)	0.07
IDAA1C 6 months	121	9.0 (8.0-9.8)	9.4 (8.3-10.2)	0.17
IDAA1C 12 months	121	9.5 (8.6-10.5)	9.9 (9.0-10.9)	0.16
IDAA1C 24 months	120	10.0 (8.9-11.2)	10.2 (9.5-11.2)	0.34

Can we safely use Alum-formulated GAD65 to induce immune tolerance in children at high risk for diabetes (Paper V)?

Baseline characteristics of the study

During screening, 54 children aged 4-17.8 years were screened for study participation. Two screened children were not positive for GADA and one additional autoantibody, one child was diagnosed with T1D and one parent withdrew consent prior to randomization. Hence, a total of 50 children were eligible for the study and consented to participate. The children were randomized 1:1 to GAD-alum or placebo treatment. The median age of the participants was 5.2 years, 27 participants (54%) were boys, and 16 participants (32%) had a first-degree relative with T1D. No difference in participant characteristics at baseline was seen between the treatment and placebo groups regarding sex, BMI, age at randomization, first-degree relative, HLA DQ risk group or autoantibody stratum (2 positive autoantibodies; 3 positive autoantibodies). Regarding glucose metabolism at baseline, no discernible difference could be seen between the randomization groups (Table 13.).

Table 13. Baseline characteristics of the DiAPREV-IT study

		GAD-ALUM n=25	Placebo n=25	p
Age at randomization (mean, range)		6.0 (4.1-15.1)	5.0 (4.0-17.9)	0.152
BMI Z-score (mean, SD)		0.22 (0.75)	0.36 (1.03)	0.570
Sex	Male	14 (56%)	13 (52%)	0.777
Population source	FDR	9 (36%)	7 (28%)	0.544
Study	DiPiS TEDDY TrialNet	10 (40%) 11 (44%) 4 (16%)	6 (24%) 17 (68%) 2 (8%)	0.329
Stratum	2 positive AAb 3-6 positive AAb	7 (28%) 18 (72%)	7 (28%) 18 (72%)	>0.999
Glucose tolerance	Impaired	13 (52%)	13 (52%)	>0.999

The median follow-up time was 4.92 years (0.47-5.01 years). One child left the study after 4.25 years of follow-up. All other children were followed for five years or until diabetes diagnosis.

Is Alum-formulated GAD safe to use in children with a high risk of T1D who are positive for GAD autoantibodies?

During study follow-up, a total of four serious adverse events were recorded. One of these events was hospital-treated pneumonia, three events were upper limb

fractures, and no events were deemed to be related to the study. An overwhelming majority of the total 878 reported adverse events in the study were classified as mild. No difference regarding the number of adverse events was seen between the treatment and placebo groups. Adverse events that were possibly related to the study drug administration were rare (12/878; 1.5%) and transient. The study drug-related adverse events included vomiting, headache, fever, nausea, sore throat, diarrhea, stomach ache and dizziness. Injection site reactions, all mild or moderate, were reported by 48/50 participants, with the same frequency being reported by the placebo and treatment groups (p=0.77).

No differences in hematology, electrolytes or liver enzymes were seen between the treatment and placebo groups during follow-up. No rapid decreases in C-peptide levels, as a sign of C-peptide collapse, were seen during follow-up. GADA titers increased in the treatment group, compared to the placebo group (p=0.001), and were significantly different up to 27 months after treatment (Figure 25.). The titers of the other autoantibodies (IA-2A, IAA, ZnT8R/W/WA, TPOA, ThglA, tTGA) analyzed during follow-up were similar between the treatment and placebo groups.

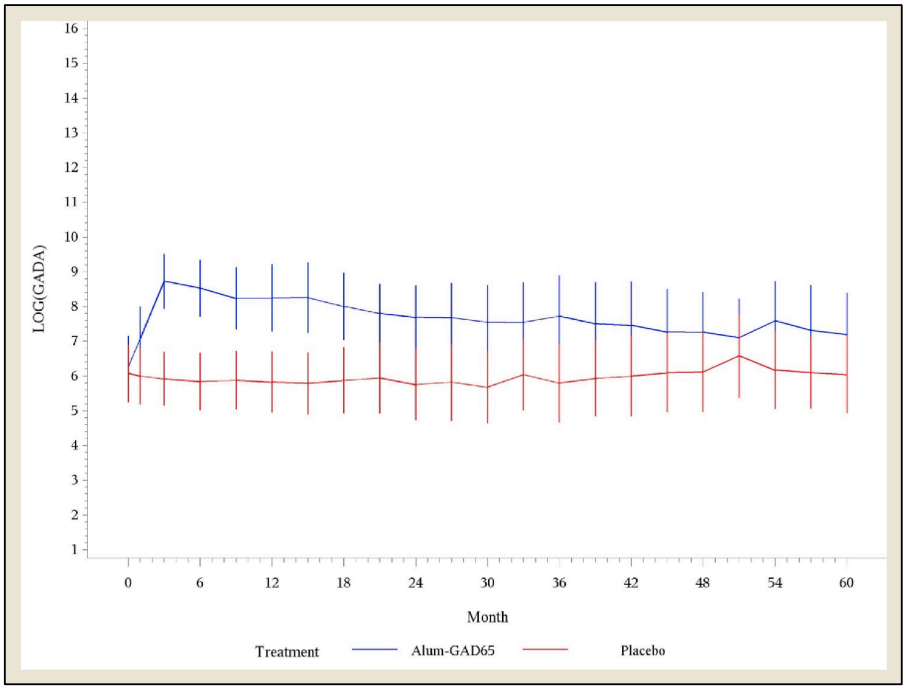

Figure 25. GADA titers in relation to treatment group during follow-up.
Control group (Red line); Treatment group (Blue line)

Does GAD-Alum affect the progression to a T1D diagnosis?

A total of 18 children (36%) were diagnosed with T1D during the five-year follow-up (170-1,830 days after the first injection). Impaired glucose metabolism at the baseline visit in the study was associated with a higher rate of progression to diabetes in comparison to children with normal glucose metabolism at baseline (p=0.013). No difference in progression to T1D was seen for first-degree relatives

(p=0.93) or in relation to the HLA risk group (p=0.21). No difference in progression to T1D could be seen between the autoantibody stratums (2 versus 3-6 autoantibodies) if autoantibodies to the ZnT8 transporter were treated as 3 separate autoantibodies (ZnT8R/W/QA) (p=0.038). However, if these autoantibodies were treated as a single autoantibody (ZnT8A), children with three or four autoantibodies progressed faster than children who were positive for only two autoantibodies (p=0.061).

No difference in the time to T1D diagnosis could be seen for the total treatment group (p=0.57) or for the stratum groups with two autoantibodies (p=0.96) or three to six autoantibodies (p=0.628). The time to diabetes diagnosis was also not affected by treatment in the group with impaired glucose metabolism at baseline (p=0.376) or the group with normal glucose metabolism (p=0.359). No difference in time to diagnosis could be seen between boys and girls (boys p=0.079; girls p=0.400).

In a univariate Cox regression model, no effect on progression to T1D could be seen for treatment with GAD-alum (HR 0.77; 95% CI 0.30, 1.94; p=0.574). In the multivariate model, no effect on progression to T1D could be seen for GAD-Alum (HR 1.15; 95% CI 0.31, 7.09; p=0.627). Female sex was the only significant covariate in the model (HR 3.69; 95% CI 1.02, 13.32; p=0.046), while first-degree relative, baseline impaired glucose tolerance, baseline FPIR, baseline AUC C-peptide, and autoantibody stratum were not significant (Table 14.).

Table 14. Hazard ratios for relevant covariates in the multivariate analysis.

	Hazard ratio	95% CI	p
Gad-Alum treatment	1.15	0.38, 3.51	0.800
Sex (girl)	3.69	1.02, 13.32	0.046
Age at randomization	0.86	0.57, 1.31	0.492
First-degree relative	1.21	0.41, 3.58	0.729
IGT at baseline	1.48	0.31, 7.09	0.627
FPIR at baseline	1.02	0.94, 1.02	0.245
C-peptide AUC at baseline	0.68	0.09, 5.33	0.717
Stratum (2 antibodies)	0.26	0.05, 1.47	0.128

FPIR, First phase insulin release; IGT, impaired glucose tolerance; AUC, area under the curve

Discussion

General discussion

The search for risk factors for T1D has been pursued for several decades, and we still only partially understand the multifactorial pathogenesis of the disease. In this thesis, we have examined the correlation between umbilical cord blood islet antibodies and T1D risk (Paper I). IA-2 autoantibodies increased the risk, even after adjustment for possible confounding factors. The maternal origin of cord blood autoantibodies has been established in previous studies[336,337]. However, the effect on T1D risk is equivocal. Previous studies have shown decreased[337], neutral[338] and increased risk[339,340]. The pathophysiological basis for this effect is also unclear. It is possible that in-utero exposure to maternal autoantibodies could affect the fetal immune system. This effect may also be a sign of exposure to maternal immune system activation.

We also investigated medication use of young children below 2.5 years of age (Paper III). The use of ANAPs is common in children, but few publications have investigated its prevalence. In particular, APAP has been investigated in conjunction with other immune mediated diseases, especially childhood asthma[341], and also in relation to autism[342]. Our results show widespread use of ANAP in this young cohort and reveal significant differences between the study sites. With regard to IA risk at six years of age, the risk does not appear to be increased in children who are exposed to ANAP, including NSAIDs or APAP. The same results apply after stratifying for age of exposure and total exposure. A weak correlation exists between APAP and IA at age three years, but only with concomitant fever. This effect was not seen in any of the other analyses. Thus, this result must be interpreted with caution. It is possible but highly unlikely that this result represents a true risk increase.

That increased stress influences T1D risk has long been proposed. In this thesis, we present data supporting the idea that parental experiences before the child turns two years old predict increased risk of T1D (paper II). Similar results have been reported by the ABIS study, in which severe life events predicted an increased T1D risk[343], although it was not possible to adjust for HLA-related risk. Other previous studies have provided equivocal results[344,345]. However, comparisons with these earlier studies are not easy because both the study endpoints and the background data differ significantly between studies.

The study of rare events, such as severe life events, is difficult, and the results must be interpreted with caution and repeated in other cohorts. That we are able reproduce similar results strengthens the concept that early life stress is an immune modulator with possible effects on immune tolerance. The analyzed parental discord and parenting stress indices failed to show any correlation with T1D risk. This finding may be due to the true absence of a correlation but may also be attributed to the use of questionnaire data without a validated instrument. Additional analysis, including laboratory markers of stress, could further strengthen the present analysis.

The backbone of current T1D pediatric research is prospective follow-up of at-risk subjects. Several large-scale studies have been and are being performed. Understanding the impact of follow-up on children and parents becomes crucial, both for study accrual and for ethical defense of the procedure. We examined the status of the children followed in DiPiS at the time of diabetes diagnosis, in comparison to children of the same age and geographic distribution (Paper IV). All children in the follow-up cohort had received risk information regarding HLA-related risk and had been prospectively followed either every year or every three months. The follow-up group was diagnosed with fewer diabetic symptoms and with a lower HbA1c, signifying a shorter and/or milder dysglycemic period before diagnosis. Those children also presented with a lower prevalence of DKA (2% compared to 18% in the control group), which is a potentially fatal complication of insulin deficiency. Studies in the DAISY[54], TEDDY[346], DPT-1[57] and BABYDIAB[56] cohorts present the same kind of results, indicating that study children are diagnosed at an early stage of the disease. We also show that the follow-up group exhibited better glycemic control 12 and 24 months after diagnosis. This finding could be attributed to the early stage at which the children are diagnosed, but that possibility seems unlikely up to two years after diagnosis. This outcome could be the result of prolonged partial remission or improved β-cell function in children who are diagnosed early. However, recent data show no difference in the rate of C-peptide decline for children diagnosed in an asymptomatic stage[347]. No demographic or socioeconomic variables were included in this analysis, and one could argue that the families willing to participate in prospective follow-up differ from the general population.

Further study will have to be done to validate these findings and to elucidate the observed effect. Understanding our follow-up cohorts is important for both interpreting results and generalizability. The ability to demonstrate beneficial, positive, results of study participation will also ease future study recruitment and help motivate further studies of otherwise healthy children.

Screening and prospective follow-up is a prerequisite of current prevention studies. Using immune tolerance is an attractive concept for inducing self-tolerance and stopping the diabetic process. Using GAD-alum, we have demonstrated that a regimen of two subcutaneous doses in children with GADA

and at least one other islet autoantibody but not T1D can be administered safely without significant side effects (paper V). The safety of GAD-alum has been studied in newly diagnosed diabetes patients with similar results. We can now add to this knowledge regarding children before diabetes diagnosis. The efficacy results are consistent with previous studies[305,306], even though some promise has been shown in a meta-analysis[348]. The study failed to demonstrate efficacy in terms of progression to T1D or any of the surrogate markers, but the study was not fully powered to do so. The study highlights the need for larger scale trials of GAD-Alum, in which dosing and timing can be examined more closely. The current protocol has been extremely time consuming, both for the investigators and the patient's families. If safety has been established, a less demanding protocol could be approved for further studies. Using immune tolerance as part of a combination treatment is also a promising concept for future trials.

Finally, we present data on the use of ANAPs in young children in the TEDDY cohort. These results are the product of an analysis of the risk of IA and use of ANAPs. The amount of data recorded in the search for the cause of T1D is extensive and enables additional analyses to be performed, enhancing our understanding of factors related to general pediatrics. We report the widespread use of ANAPs in very young children in the TEDDY cohort, with significant differences in prevalence between the TEDDY sites. We also observed a preference for the use of APAP among first-born children in comparison to their younger siblings. The use of ANAPs was primarily related to fever or infection in the studied cohort, but a significant portion of participants also used these drugs without fever or infection, especially in the US cohort. Understanding basic patterns of use in pediatric patients is important for the pediatric community, and to or knowledge, no similar data have been published to date.

Weaknesses

There are weaknesses associated with the papers in this thesis. The statistical analysis of rare events is not easy and can be controversial. In both paper I and paper II, there are low numbers of exposures and a relatively low number of events. This factor leads to increased uncertainty in the model. Enhancing the statistical model further with a penalized model or choosing a different statistical test altogether could alleviate some of this uncertainty. For paper II, extending the data to a longer time frame would increase the background data but lead to a significant reduction in the number of participants. The analysis in paper I was performed as a group-wise comparison between the follow-up and non-follow-up groups. This approach does not take into account any differences in baseline variables that may influence the result. Ongoing extended analysis of these data up to five years after diagnosis should include baseline parameters, as well as a better

measurement of residual C-peptide and growth parameters to be included in a longitudinal model. In paper III, the statistical limitation is the opposite: a very widespread exposure in the cohort, with the exposed groups surpassing the non-exposed group, leading to uncertainty in the statistical model. All papers related to the DiPiS study rely partially on questionnaire data recorded by the parents at home, even though the data are collected prospectively. This approach is associated with some level of recall and measurement bias, as well as inconsistent results from year to year. Missing data in the questionnaires also limit the number of subjects available for multivariate analysis. In the TEDDY study, all data are collected at nurses' visits, but the analysis still relies on parental recollection and interpretation. Although extensive data were collected, uncertainties remain regarding the exact reasons for the use of ANAP outside of fever and infections.

In paper II, questionnaire data are used to assess life events and to compute the two stress indices. Using a validated questionnaire to assess family stress would have improved the validity of the analysis. An additional weakness in papers II and IV is the lack of supporting laboratory data. The inclusion of C-peptide values and stress biomarkers, respectively, would have improved the interpretation of the results in these papers, but that approach was not part of the original study plan and was thus not available for the analysis. A general weakness of papers I – IV is the high-risk composition of the cohort. It is possible that the risk factors described in these HLA-derived high-risk cohorts are not valid outside of this group. This factor is a problem that both DiPiS and TEDDY share with several other cohorts. If T1D, and IA to some extent, are used as endpoints, this limitation is in many ways unavoidable due to both time and monetary constraints.

Future issues

Our pursuit of the causes and pathogenesis of T1D has been ongoing for a long time. Still, even though our knowledge is significantly better, large pieces of the puzzle are missing. Prior methods of research have been painstaking and slow, relying on long-term follow-up with T1D as the study endpoint. Ongoing studies, like the TEDDY study, that use IA as an earlier, alternate endpoint, together with a large, multinational study cohort and intensive and thorough follow-up hold great promise for achieving the goal of identifying the environmental determinants of diabetes and improving our understanding of the natural history of T1D. Using alternate endpoints besides diabetes diagnosis will be important in the future for both monetary and timing reasons. In particular, intervention trials will benefit from the use of alternate endpoints when new treatment regimens and combinations are used. For intervention trials, the use of combination treatments for induction and maintenance treatment could build on the success of pediatric

oncological treatment. Finding an acceptable level of side effects for long-term treatment, which is a better option than T1D, is crucial.

The amount of research surrounding risk factors, natural history, genetic and epigenetic factors is expanding to a level that is hard to grasp. T1D is truly a multifactorial disease. Personalizing this data for the individual will be harder as our knowledge increases. Machine learning and enhanced algorithms hold promise for improving our understanding of risk factors and extracting data from large datasets.

One of the central issues that must be addressed is the severity of T1D and establishing the extremely high risk that is associated with multiple islet autoantibody positivity. Today, T1D is perceived as a fairly benign disease, for which patients must take their insulin, do their glucose test, and otherwise live a normal life. Many, if not all, families living with T1D would disagree with that description. T1D is a disease that encompasses every aspect of life and places an overwhelming burden on parents and children. In addition, even if patients reach the difficult goal of glycemic control, they remain at an increased risk of vascular damage and a shortened life span. In ethical discussions, these aspects must also be considered. It is easy to say no to involving children in research, deeming it unethical. However, it may be the failure to learn more and research diseases that affect children that is unethical.

Conclusions

- IA-2A cord blood autoantibodies and early severe life events during the child's first two years of life potentially increase the risk of T1D in children (Paper I, Paper II).
- ANAP use in children below 2.5 years of age does not predict the risk of IA at age six years, although a weak correlation with IA was observed at age three years (Paper III).
- Children enrolled in follow-up in the DiPiS cohort are less affected by diabetes symptoms and have a significantly lower prevalence of DKA at diagnosis (Paper IV).
- Children enrolled in follow-up in the DiPiS cohort have better glycemic control up to two years after diagnosis, compared to children who are not enrolled in follow-up (Paper IV).
- Treatment with GAD-Alum does not present any safety concerns when treating multiple islet autoantibody-positive children who have not yet been diagnosed with T1D (Paper V).
- GAD-Alum does not show efficacy in terms of delaying or preventing T1D diagnosis in multiple islet autoantibody-positive children in this small-scale trial (Paper V).
- The use of ANAP in children below 2.5 years is widespread, and significant between-country differences exist (Paper III).

Summary in Swedish

Vad är typ 1 diabetes

Typ 1 diabetes är en av de vanligaste kroniska sjukdomarna som drabbar barn. Sjukdomen orsakas av att kroppen attackerar de så kallade Langerhanska cellöarna i bukspottskörteln via en autoimmun reaktion. De egna cellerna uppfattas alltså som främmande och förgörs. Detta leder till en oförmåga att producera insulin, det hormon som styr vår blodsockernivå i blodet. Denna autoimmuna process kan i nuläget varken förhindras eller stoppas. När sjukdomen är fullt utvecklad krävs insulininjektioner vid ett flertal tillfällen dagligen för att blodsockret skall hållas på rätt nivå samt även upprepade kontroller av blodsockernivån.

Vi vet nu att utvecklingen av typ 1 diabetes startar långt innan några symtom visar sig. I flertalet fall kan tecken i blodet ses åtskilliga år tidigare, inte sällan så tidigt som innan 1 års ålder. Sårbarheten för att vara mottaglig för typ 1 diabetes är genetisk och framför allt relaterad till ett område som kallas HLA, som är en viktig del av immunförsvaret. Den så kallade HLA-genotyp en individ har bestämmer ungefär hälften den medfödda genetiska risken för typ 1 diabetes. Den andra hälften av den medfödda risken verkar komma från ett flertal andra gener som vi i nuläget inte fullt ut har utforskat. Omgivningsfaktorer är också viktiga för risken att utveckla typ 1 diabetes. Vi vet idag att vissa omgivningsfaktorer verkar påverka diabetesrisken, däribland virusinfektioner. Vår förståelse är dock på inget sätt fullständig och vi lär oss ständigt mer om hur risken för typ 1 diabetes samverkar, både med vår genetik men också med vår omgivning.

Bedömning av risk för typ 1 diabetes

Det första sättet att avgöra risk för typ 1 diabetes är genom tester för HLA-genotyp. De varianter som medför högst risk för typ 1 diabetes är dock vanliga i svensk befolkning och någon av dem finns hos en stor del av befolkningen. Senare i livet kan dock en av de sex diabetesrelaterade autoantikropparna utvecklas som tecken på en sjukdomsprocess. Dessa antikroppar är immunförsvarets målsökande missiler, i detta fallet riktade mot olika delar av de insulinproducerande cellerna. Autoantikropparna har namn efter vilken struktur de är riktade mot och kallas IAA, IA-2A, GADA, ZnT8 (WA/RA/QA). I de flesta fall uppkommer en variant av dessa för att senare följas av ytterligare. En enstaka autoantikroppsvariant medför en lätt ökad risk för typ 1 diabetes, men denna kan också försvinna igen och med den ökade diabetesrisken. De barn som utvecklar mer än en

autoantikropp har dock en mycket hög risk för typ 1 diabetes inom 5–10 år. Vissa hävdar att det till och med är att likna vid ett sjukdomstillstånd, även om inga symtom än har visat sig.

För att ytterligare klargöra risken för att insjukna kan vi bedöma kroppens förmåga att hantera socker genom både orala glukosbelastningar, där en bestämd mängd sockerlösning dricks, eller intravenösa glukosbelastningar, där socker ges direkt i blodet. I samband med dessa kan man månader, och ibland år, innan diabetesdiagnosen sätts, se en långsam successiv försämring i insulininsöndring och blodsockerkontroll.

Hur har data till studierna samlats in?

I vårt arbete har vi framförallt använt två stora grupper av barn som följts sedan födseln. I Skåne screenades 35 683 barn mellan september 2000 och augusti 2004 vid födseln avseende diabetesrisk. De närmre 8000 barn som bedömdes ha ökad risk erbjöds att delta i uppföljning fram till barnen fyllde 15 år. Knappt 3900 barn har deltagit i DiPiS studiens (DiabetesPrediktion i Skåne) uppföljning årligen från två års ålder med blodprover och frågeformulär. Barn som utvecklar tecken på autoimmunitet har följts var tredje månad. TEDDY-studien (The Environmental Determinants of Diabetes in the Young) screenade närmre 415 000 barn vid sex olika centra i Sverige, Finland, Tyskland och USA. Cirka 21 500 barn hade ökad risk och erbjöds vara med i studien varav 8700 tackade ja. TEDDY studiens uppföljning började vid 3 månaders ålder och är väldigt omfattande. Prover från såväl blod, urin, avföring, hår, nässekret, hushållets vatten med mera samt redogörelser för faktorer i barnens liv så som sjukdomar, mathållning och medicinering och fysisk aktivitet samlas in. Barnen följs upp var tredje månad till fyra års ålder och för de som inte utvecklat tecken till autoimmunitet glesas då besöken ut till var sjätte månad.

Vilka är resultaten?

Denna avhandling försöker förbättra vår kunskap om typ 1 diabetes på flera områden i sjukdomsprocessen. Vårt första mål var att undersöka riskfaktorer relaterade till navelsträngsblod. Vi tog prover från navelsträngen vid barnets födsel och undersökte vilka barn som hade diabetesrelaterade autoantikroppar från mamman. Risken för de barn som hade IA-2 autoantikroppar i navelsträngsblodet ter sig i denna studie vara cirka sju gånger högre än för barnen utan antikroppar. De övriga antikropparna som analyserades verkade inte påverka diabetesrisken (IAA och GADA). I nuläget har vi ingen förklaring på varför detta diabetesrisken påverkas men hoppas att detta kan bekräftas och klargöras i framtida studier.

Vi har därefter undersökt hur användningen av febernedsättande och smärtlindrande läkemedel innan 2,5 års ålder ser ut hos TEDDY-studiens barn. Detta rör sig framförallt om paracetamol (t. ex. Alvedon och Panodil) och NSAID-preparat/ibuprofen (t. ex. Ipren, Nurofen, Treo eller Albyl). Vi undersökte därefter

om användningen av dessa preparat medförde en ökad risk för utveckling av autoantikroppar vid sex års ålder. Våra resultat visar inte på någon riskökning för ö-cellsautoimmunitet vid sex års ålder även om en svag koppling finns vid tre års ålder. Vi kan även beskriva stora skillnader i hur mycket och ofta dessa preparat används i de olika länderna. Att en stor andel av förbrukningen hos dessa väldigt unga personer sker utan varken feber eller infektion var även en nyhet för oss.

Den sista faktorn avseende diabetesrisk vi har undersökt är hur föräldrars stress och negativa livshändelser för familjen innan 2 års ålder påverkar diabetesrisken. Vi använde oss av svar från frågeformulär vid 2 månader och 2 års ålder. För att lättare kunna bedöma föräldrarnas stressnivåer utformades två olika index, dels faktorer talande för föräldrarnas oro och dels för hur stressande relationen till barnet var. Inget av dessa index, och därmed svaren gällande föräldrars stress, ter sig påverka barnets risk för typ 1 diabetes. Vi har även undersökt om negativa livshändelser påverkar diabetesrisken. Detta kan röra sig om våld i familjen, arbetslöshet, separation, sjukdom mm. Våra data visar en ökad risk för typ 1 diabetes hos de barn vars familjer upplevt en negativ livshändelse innan barnet fyllt två år. I nuläget kan vi inte fullt förklara vad som ligger bakom denna effekt men en förklaring kan vara att stresshormoner påverkar immunförsvaret och därmed ökar risken för autoimmun sjukdom.

Att vara med i en studie under barndomen är en speciell situation för barn och familjen. Vi ville undersöka om de barn som deltagit i DiPiS-studien och som fått information om sin diabetesrisk skiljde sig från barn som ej deltagit, när de får typ 1 diabetes. Vi kan visa att barn som deltagit i DiPiS har väsentligt färre symtom vid diagnos och dessutom har haft lägre nivåer på sitt blodsocker innan diagnos. Det är även färre som har syraförgiftning, ketoacidos, när de får sin diagnos. Ketoacidos är ett mycket allvarligt tillstånd som i vissa fall leder till intensivvård och kan vara dödligt. När vi sedan följer dessa två grupper barn under två år ser vi att de barn som deltagit i studien har bättre blodsockerkontroll både ett och två år efter diagnos. Det är glädjande att vi kan diagnosticera studiebarnen tidigt utan att de hunnit bli allvarligt sjuka, vilket kan både minska chocken vid diagnos och även korta vårdtiden. Att blodsockerkontrollen är bättre upp till två år efter diagnos kan bero på att större andel insulinproducerande celler kvarstår vid en tidig diagnos, men kan också bero på att familjen är bättre förberedd på barnets sjukdom. Vi kommer undersöka detta närmare för att veta bättre.

Slutligen visar vi resultat från en studie som försöker stoppa eller förlångsamma sjukdomsprocessen. Vi har undersökt 50 barn med hög risk för diabetes och minst två autoantikroppar. Hälften av dessa barn har fått behandling vid två tillfällen med GAD-Alum och hälften fick overksam substans (placebo). Detta preparat skall försöka vänja kroppen vid GADA, en av de autoantikroppar som är involverade i utvecklingen av typ 1 diabetes. Studiens huvudmål var att avgöra om det var säkert att ge detta preparat till barn utan diabetes. Inga allvarliga biverkningar rapporterades och vi kan visa att preparatet kan ges säkert på detta

sätt. Vi undersökte även om GAD-Alum påverkade risken för att få diabetes eller hur lång tid det tog att få diabetes. Våra resultat visar att det inte verkar ha någon effekt på diabetesutveckling. Gruppen studiepersoner är dock väldigt liten och det blir därmed svårt att visa en eventuell effekt rent statistiskt jämfört med om studien varit större.

Sammanfattning

Vi kan visa att autoantikroppar i navelsträngsblod mot IA-2 och allvarliga livshändelser under barnets två första levnadsår verkar öka risken för typ 1 diabetes. Däremot ser vi ingen påverkan på risken för förstadiet till typ 1 diabetes, autoimmunitet, relaterat till intag av smärtstillande/febernedsättande medel hos barn under 2,5 år. De barn vi följer i DiPiS-studien diagnosticeras med diabetes tidigt i sjukdomsförloppet och är också mindre påverkade vid sin diagnos. Deras blodsockerkontroll är bättre både vid 1 och 2 års uppföljning efter diabetesdiagnosen. Slutligen är behandling med GAD-Alum, till barn med autoantikroppar men inte diabetes, säkert men verkar inte påverka risken för diabetes.

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Paper I

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SHORT COMMUNICATION

Cord blood insulinoma-associated protein 2 autoantibodies are associated with increased risk of type 1 diabetes in the population-based Diabetes Prediction in Skåne study

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Abstract
Aims/hypothesis The aim of this study was to examine the effect of cord blood autoantibodies on the risk for type 1 diabetes in children followed prospectively from birth.
Methods The Diabetes Prediction in Skåne (DiPiS) study consists of 35,853 children from the general population born during 2000–2004. Samples were collected at birth and analysed for HLA genotypes and autoantibodies to glutamate decarboxylase 65 (GAD65), insulin and insulinoma-associated protein 2 (IA-2). After adjusting for HLA, sex, maternal age and parental type 1 diabetes, independent associations with risk of diabetes were assessed using multivariate Cox proportional hazards models.
Results In total, 151 children (0.4%) had developed type 1 diabetes by the end of 2013 at a median age of 5.8 years (0.8–12.2 years). In the multivariate analysis, the presence of IA-2 autoantibodies (IA-2A) in cord blood (HR 6.88, 95% CI 1.46,32.4; p=0.003), but not maternal diabetes (HR 1.38, 95% CI 0.24,7.84; p=0.71), was associated with risk of developing type 1 diabetes. No increased risk could be seen for the presence of autoantibodies to GAD65 or insulin.
Conclusions/interpretation Our study indicates that the presence of cord blood IA-2A superimposes maternal diabetes and other cord blood islet autoantibodies as a predictor of type 1 diabetes development in the child. These findings may be of significance for future screening and study protocols on type 1 diabetes prediction.

Keywords GAD65 autoantibodies · IA-2 autoantibodies · Insulin autoantibodies · Paediatric · Prediction · Type 1 diabetes

Abbreviations

BDD	Better Diabetes Diagnosis
DBS	Dried blood spot
DiPiS	Diabetes Prediction in Skåne
GADA	Glutamic acid decarboxylase 65 autoantibodies
IAA	Insulin autoantibodies
IA-2A	Insulinoma-associated protein 2 autoantibodies

Introduction

Early prediction of type 1 diabetes is crucial in the attempts at preventing or stalling the disease. Identifying risk factors for islet autoimmunity and factors accelerating the autoimmune process will enable more successful study enrolment and treatment attempts.

Islet autoantibodies are found in 3–5% of cord blood samples from newborns in the general population [1], with an even higher proportion found, as expected, in offspring of

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mothers with type 1 diabetes [2]. Previous studies, mainly recruiting children with first-degree relatives with type 1 diabetes, have investigated the significance of cord blood autoantibodies with contradictory results [3, 4].

As only 10–13% of newly diagnosed children and young adults with type 1 diabetes have a first-degree relative with the disease it is difficult to make predictions for the general population based on analyses of these subgroups. In the Diabetes Prediction in Skåne (DiPiS) study, children from the general population are followed from birth with the aim of identifying risk factors and predictive markers of type 1 diabetes. In the present study we had the opportunity to examine the impact of cord blood autoantibodies on risk of type diabetes.

Methods

Study population and participants In the southernmost part of Sweden, cord blood of children born between September 2000 and August 2004 was analysed for HLA-DQ genotypes, glutamate decarboxylase 65 autoantibodies (GADA), insulin autoantibodies (IAA) and insulinoma-associated protein 2 autoantibodies (IA-2A) in the DiPiS study [5]. When the child reached 2 months of age the parents were asked to fill out a written consent form and a questionnaire regarding family history of diabetes, birthweight, gestational age and perinatal infections [5]. The study protocol and collection of data are illustrated in ESM Figure 1.

By the end of 2013, the DiPiS cohort had reached 9–13 years of age and 151 children had been diagnosed with type 1 diabetes. New patients are registered via the Better Diabetes Diagnosis (BDD) study, covering an overwhelming majority of new diabetes cases in Sweden. The regional ethics review board in Lund, Sweden approved the study.

HLA genotyping HLA was analysed on dried blood spot (DBS) filters as described in detail elsewhere [6]. In the current analysis, HLA was classified as HLA-DQA1*0501-DQB1*0201 (DQ2) or HLA-DQA*0301-DQB1*0302 (DQ8) and stratified into the following four risk groups: (1) DQ 2/8; (2) DQ8/8 or 8/X; (3) DQ2/2 or 2/X; (4) DQ X/X (X is neither DQ2 nor DQ8).

Cord blood autoantibodies DBS eluates were incubated with labelled antigen to GAD65 and IA-2, and autoantibody-bound labelled antigen was separated from free with Protein A-Sepharose (Amersham Biosciences, Uppsala, Sweden). Positive samples (combined GADA and IA-2A analysis >99th percentile) were reanalysed in separate assays for GADA and IA-2A. IAA was screened in serum in a microassay. All samples above the 99th percentile were reanalysed to correct for nonspecific binding. The exact procedure has been described elsewhere [7].

Statistical methods Statistical analyses were performed using SPSS (version 21, SPSS, Chicago, IL, USA) and R version 3.03 using the survival package (R Core Team [2014] R Foundation for Statistical Computing, Vienna, Austria). HRs with corresponding 95% CIs, as estimated by univariate Cox proportional hazards models, were used to identify factors with significant influence on risk of type 1 diabetes in the child. A multivariate Cox proportional hazards model tested for an independent association of each autoantibody with the child's diabetes risk while also adjusting for HLA-DQ, sex, maternal age and parental type 1 diabetes. A separate baseline hazard was fitted for each HLA risk group. HRs for HLA genotype were calculated in a multivariate model without stratification. Multivariate analysis included the following variables: cord blood IA-2A, IAA and GADA (categorised as positive if above the 99th percentile, treated as categorical variables), gestational week, relative birthweight percentile, infection during pregnancy, HLA risk group, sex, maternal and paternal type 1 diabetes, and mother's age at delivery.

Results

Baseline characteristics A total 35,683 cord blood samples were collected in 48,058 children born during the study period. Parents of 25,392 children returned the 2-month questionnaire. Of the responders to the questionnaire, 190 children had a mother with type 1 diabetes and 985 had gestational diabetes. Paternal type 1 diabetes was reported in 285 of children (ESM Table 1). As of 31 December 2013, 151 children had been diagnosed with type 1 diabetes with a median age at diagnosis of 5.8 years (0.8–12.2 years). In total, 12 children had a parent with type 1 diabetes (8%); eight fathers and four mothers. Autoantibody status and demographics at diagnosis are shown in (ESM Table 1).

Autoantibodies in cord blood and their influence on T1D risk Autoantibodies against GAD65, IA-2 and insulin were found in 295 (0.8%), 78 (0.2%) and 509 (1.4%) cord blood samples, respectively. A total of 44 samples (0.1%) were positive for both IA-2A and GADA, and 25 samples (0.07%) were triple positive. Contingency tables with autoantibody status and overlap with maternal type 1 diabetes are shown in ESM Tables 2–4. In children of diabetic mothers the presence of IA-2A in cord blood was associated with increased risk for the development of type 1 diabetes in the

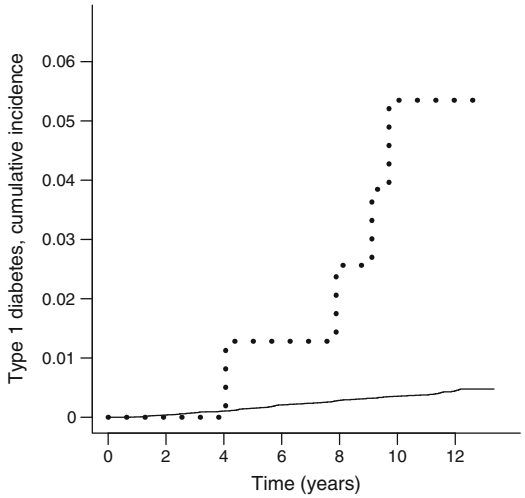

Fig. 1 Progression to type 1 diabetes related to IA-2A cord blood positivity (solid line, IA-2 negative; dotted line, IA-2 positive)

child (Fisher's exact test $p=0.037$; ESM Table 2). However, in children with IA-2A present in cord blood the presence of diabetes in the mother was not associated with the development of type 1 diabetes in the child (Fisher's exact test $p=1$; ESM Table 3).

In a multivariate model with autoantibodies and parental type 1 diabetes included, only IA-2A was a predictor of the child's risk for the development of diabetes. The risk was significant in both a univariate model (HR 14, 95% CI 5.0,37; $p<0.001$) and a multivariate model (HR 6.4, 95% CI 1.8,22; $p=0.003$) (Fig. 1). GADA and IAA alone showed evidence of association with risk of type 1 diabetes, but not after adjustment for maternal type 1 diabetes status at birth (GADA HR 1.5, 95% CI 0.5,4.8; $p=0.48$; IAA HR 1.1, 95% CI 0.4,3.4; $p=0.82$; Table 1).

HLA-DQ derived risk and parental diabetes The risk profile with HLA_DQ genotypes was similar to that of previous studies (Table 1).

Having a father with type 1 diabetes was associated with an increased risk for the development of diabetes in the child (HR 3.4, 95% CI 1.6,7.0; $p<0.001$). Maternal diabetes was associated with the child's risk of developing type 1 diabetes (HR 3.5, 95% CI 1.3,9.5; $p=0.014$) but not after adjusting for the presence of IA-2A (HR 1.6, 95% CI 0.3,7.0; $p=0.60$; Table 1).

Discussion

In this population-based prospective study, we report that the presence of IA-2A in cord blood increases the risk for the development of type 1 diabetes in the child, and this risk seems to be unrelated to the diabetes status of the mother.

It has been well established that islet autoantibodies, and specifically IA-2A, measured during childhood predicts the

Table 1 Association between selected factors and diagnosis of type 1 diabetes in the DiPiS study cohort

	Univariate HR (95% CI)	*p* value	Multivariate[a] HR (95% CI)	*p* value	Multivariate[b] HR (95% CI)	*p* value
Parental type 1 diabetes						
Mother	3.50 (1.30, 9.47)	0.014	1.51 (0.32, 7.03)	0.60	1.38 (0.24, 7.84)	0.71
Father	6.21 (3.02, 12.8)	<0.001	3.39 (1.64, 7.02)	<0.001	3.70 (1.77, 7.77)	<0.001
IA-2A-positive	13.5 (5.02, 36.6)	<0.001	7.73 (1.94, 30.7)	0.003	6.88 (1.46, 32.4)	0.015
IAA-positive	2.41 (0.98, 5.88)	0.053	0.75 (0.19, 3.00)	0.82	0.65 (0.14, 3.08)	0.58
GADA-positive	3.44 (1.27, 9.29)	0.015	1.15 (0.33, 4.01)	0.48	1.11 (0.27, 4.50)	0.88
Relative birthweight, quartile	1.08 (0.91, 1.29)	0.38			1.02 (0.85, 1.23)	0.82
Infection during pregnancy	0.85 (0.52, 1.39)	0.51			0.82 (0.47, 1.43)	0.48
Age of mother	1.01 (0.98, 1.04)	0.63			1.00 (0.95, 1.04)	0.94
Gestational length	0.86 (0.80, 0.93)	<0.001			0.85 (0.73, 0.99)	0.04
Sex	0.89 (0.65, 1.23)	0.48			0.89 (0.58, 1.34)	0.57
HLA risk group						
DQ 2/8	18.2 (13.1, 25.3)	<0.001	42.4 (23.3, 77.2)	<0.001		
DQ 8/8 or 8/X	3.48 (2.42, 5.01)	<0.001	10.9 (5.74, 20.7)	<0.001		
DQ 2/2 or 2/X	2.02 (1.30, 3.16)	0.002	8.51 (4.20, 17.2)	<0.001		
DQ X/X (ref)	1.0 (ref)	ref	1.0 (ref)	ref		

[a] Multivariate model including maternal type 1 diabetes, paternal type 1 diabetes, IA-2A, GADA and IAA and stratified for HLA-DQ. HLA HR calculated in maltivariate model without stratification

[b] Multivariate model including all available variables and stratified for HLA-DQ

onset of type 1 diabetes. However, few studies have investigated the association between diabetes risk and the presence of cord blood autoantibodies, and the existing data are somewhat conflicting. Several studies of children born to mothers with type 1 diabetes found that cord blood autoantibodies were of maternal origin [1, 8, 9]. Moreover, the maternal origin of cord blood autoantibodies was confirmed in a study of non-diabetic mothers [7], but further studies on this matter are needed. In the offspring of mothers with type 1 diabetes, cord blood autoantibodies have been reported to increase the risk for development of diabetes in the child [10], but also to have no effect [4] or even be protective [3]. In a retrospective case-control study of cord blood sera from children diagnosed before the age of 15 years, the presence of cord blood autoantibodies was reported to increase the risk of the child developing type 1 diabetes, even after excluding mothers with diabetes [10]. Unfortunately, no analysis of IA-2 was performed, with islet cell antibodies being used instead [10]. It cannot be excluded that IA-2A may have contributed to the risk of cord blood autoantibodies for type 1 diabetes in that study. A recent Danish study reported an increased risk of type 1 diabetes in children born with cord blood autoantibodies, but no separate analysis was performed for IA-2A and GADA [9].

One of the major strength of our analysis is that the data originate from a large-scale screening programme of the general population, with 70% of all children born in the Skåne region sampled at birth. Our data regarding the prevalence of type 1 diabetes in this cohort, now aged 9–13 years, are considered complete. The well-covered study area in Sweden, with five paediatric clinics and validation against the BDD study, minimises the risk of having missed new cases.

Our study is limited by reliance on questionnaire data regarding some pre- and perinatal factors. We can, therefore, assume that the data suffer from recall bias. Missing data from the 2-year questionnaire affect the number of patients eligible for multivariate analysis. However, we were still able to use data from 17,287 children and 89 type 1 diabetes patients. The number of children who were autoantibody-positive at birth was small, as was the number of children of mothers with type 1 diabetes. This introduces some uncertainty into the statistical analysis.

In conclusion, our study indicates that the presence of cord blood autoantibodies to IA-2, but not GADA or IAA, increases the risk of developing type 1 diabetes compared with the general population. The increased risk of maternal diabetes disappears after adjusting for IA-2A in cord blood, suggesting that IA-2A may be the primary risk factor. Further studies are needed to confirm this finding.

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Contribution statement ML analysed the data and wrote the manuscript. KL was involved in the study design and critically revised the manuscript for important intellectual content. CL provided statistical support, database management and contributed to and edited the manuscript. HEL designed the study, was involved in data collection, interpreted data and contributed to and edited the manuscript. All authors gave final approval of the version to be published. ML is responsible for the integrity of the work as a whole.

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Paper II

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BMC Pediatrics

RESEARCH ARTICLE Open Access

Analgesic antipyretic use among young children in the TEDDY study: no association with islet autoimmunity

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Abstract

Background: The use of analgesic antipyretics (ANAP) in children have long been a matter of controversy. Data on their practical use on an individual level has, however, been scarce. There are indications of possible effects on glucose homeostasis and immune function related to the use of ANAP. The aim of this study was to analyze patterns of analgesic antipyretic use across the clinical centers of The Environmental Determinants of Diabetes in the Young (TEDDY) prospective cohort study and test if ANAP use was a risk factor for islet autoimmunity.

Methods: Data were collected for 8542 children in the first 2.5 years of life. Incidence was analyzed using logistic regression with country and first child status as independent variables. Holm's procedure was used to adjust for multiplicity of intercountry comparisons. Time to autoantibody seroconversion was analyzed using a Cox proportional hazards model with cumulative analgesic use as primary time dependent covariate of interest. For each categorization, a generalized estimating equation (GEE) approach was used.

Results: Higher prevalence of ANAP use was found in the U.S. (95.7%) and Sweden (94.8%) compared to Finland (78.1%) and Germany (80.2%). First-born children were more commonly given acetaminophen (OR 1.26; 95% CI 1.07, 1.49; $p = 0.007$) but less commonly Non-Steroidal Anti-inflammatory Drugs (NSAID) (OR 0.86; 95% CI 0.78, 0.95; $p = 0.002$). Acetaminophen and NSAID use in the absence of fever and infection was more prevalent in the U.S. (40.4%; 26.3% of doses) compared to Sweden, Finland and Germany ($p < 0.001$).
Acetaminophen or NSAID use before age 2.5 years did not predict development of islet autoimmunity by age 6 years (HR 1.02, 95% CI 0.99-1.09; $p = 0.27$). In a sub-analysis, acetaminophen use in children with fever weakly predicted development of islet autoimmunity by age 3 years (HR 1.05; 95% CI 1.01-1.09; $p = 0.024$).

Conclusions: ANAP use in young children is not a risk factor for seroconversion by age 6 years. Use of ANAP is widespread in young children, and significantly higher in the U.S. compared to other study sites, where use is common also in absence of fever and infection.

Keywords: Type 1 diabetes, Analgesics, Islet autoimmunity, Prospective studies

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Background

The administration of analgesic-antipyretic (ANAP) medications to children has been discussed in the literature for decades. Surveys of Canadian and American pediatricians reflect the routine use of acetaminophen and non-steroidal anti-inflammatory drugs (NSAID) for childhood fever and discomfort [1, 2]. In the 1980s, the term "fever phobia" was used to describe the parental pressure facing pediatric practitioners to manage fever [3]. Parental misconceptions often lead parents to the inappropriate management of fever in their children [4] and parents report the use of antipyretics even when there was minimal or no fever [5] as parents were frequently concerned with the need to maintain a "normal temperature" in their ill child [6]. Nevertheless, additional studies are needed to support this as evidence-based practice [7, 8]. Acetaminophen and NSAID are used widely in children, but limited data exist regarding patterns of use in countries beyond the United States, United Kingdom, France, and Canada [9].

Notably, acetaminophen has been shown to have effects on glucose homeostasis. High doses have been shown to induce hyperglycemia [10], whereas low and chronic doses can lower blood glucose in animal models [11–13]. Possible effects on asthma risk have also been investigated [14, 15]. NSAIDs have also been shown to lower blood glucose [16, 17], but have additional anti-inflammatory properties that could have an impact on the process leading up to T1D [18].

The Environmental Determinants of Diabetes in the Young (TEDDY) Study is an international, multi-center study designed to identify the environmental triggers of T1D in genetically at-risk children [19]. The aim of the current study was to describe the use of ANAP in the TEDDY study, as well as differences in relation to country, birth order (first child versus a child with older siblings) and fever status. Specifically, we sought to examine if the use of ANAP: (1) is associated with risk for islet autoimmunity (IA), (2) differs between countries, (3) is given preferentially to first-born children.

Methods

The Environmental Determinants of Diabetes in the Young (TEDDY) is a prospective cohort study funded by the National Institutes of Health with the primary goal to identify environmental causes of type 1 diabetes (T1D). It includes six clinical research centers - three in the US: Colorado, Georgia/Florida, Washington and three in Europe: Finland, Germany, and Sweden. Detailed study design and methods have been previously published [19, 20]. Written informed consents were obtained for all study participants from a parent or primary caretaker for genetic screening and participation in prospective follow-up. The study was approved by local Institutional or Ethics Review Boards (Additional file 1), and is monitored by an External Advisory Board formed by the National Institutes of Health.

Data collection

The dataset analyzed was the data received by the TEDDY Data Coordinating Center as of December 31, 2014. The total number of subjects enrolled was 8676. Analysis was restricted to confirmed HLA eligible subjects and subjects with medication information in the first 2 years of age. Out of the enrolled subjects, 134 were missing medication data and were excluded from the analysis. Information regarding first child status was missing for 919 subjects who were also excluded, leaving a total of 7623 subjects (Additional file 2).

Study visits were conducted every 3 months with the first visit occurring between 3 and 4.5 months of age. At each visit, interviewers recorded the name, reason, start date and duration of reported medications for the most recent visit interval. Parents were asked to document fever as either "Yes" or "No" for every illness entry in a "TEDDY Book." The "TEDDY Book" provided written guidance that "Yes" should only be marked for temperature equal to or greater than 38 °C or 101 °F. Approximately 18 months into the study, these choices were expanded to "Yes – measured," "Yes – not measured," and "No." The rationale for this change was to capture all uses of ANAP, even with low-grade fevers.

Each use of an ANAP was defined as an episode. Recorded medications were categorized based on active ingredient. When analyzing specific substances, all medications containing that particular substance were included. Drugs were also defined and grouped as either analgesic or non-steroidal anti-inflammatory (NSAID) (Additional file 3). Episodes were described as associated with infection and/or fever. Infection was defined as either an ICD-10 code indicating Infection (Additional file 4) or an acute illness designated as infectious within a 15 day time period of the medication date [21]. Fever was defined as either an ICD-10 code of fever associated with the medication or an acute illness associated with fever within a 15-day time period of the medication date.

Islet autoimmunity

Blood samples were drawn every 3 months between 3 and 48 months of age, and every 6 months thereafter, except for autoantibody positive children, who continued with visits every 3 months. Persistent IA was defined as positive antibodies to insulin (IAA), glutamic acid decarboxylase (GAD65), or insulinoma-associated antigen 2 (IA-2), each analyzed by radiobinding assay [22, 23], on at least 2 consecutive study visits. Two central autoantibody laboratories were used; one in the U.S. (Barbara

Davis Center for Childhood Diabetes at the University of Colorado) and one in Europe (University of Bristol). All positive islet autoantibodies and 5% of negative islet autoantibodies were confirmed in both central autoantibody laboratories. Both laboratories have previously demonstrated high sensitivity, specificity [24] and concordance. Positive results in the child that were deemed to be due to maternal IgG transmission were excluded from the IA-positive group.

Statistical methods

A Cox proportional hazards model was used to assess the impact of ANAP use in the first 90, 180, 365 days of age and 2.5 years of age in the risk of positive autoantibodies through 6 years of age. The number of infections early in life was included as a time dependent covariate [25]. Country was included as a stratification factor in the proportional hazards analyses. Additional covariates included in the model were first-degree relative [26], HLA [27], gender, ever breastfed [28, 29], probiotic use prior to 3 months of age [30], and eight different previously identified single nucleotide polymorphisms [31]. The primary variable of interest was cumulative ANAP use through 2.5 years of life as a time dependent covariate. Included covariates can be seen in Table 1.

The statistical analysis for the number of episodes per year and duration per year excluded subjects for which the first child status was missing. Subjects with a missing duration for a specific analgesic were excluded from the analysis for that analgesic. The statistical analysis of total duration per year was based on log-transformed data to better satisfy the assumptions of the linear models.

Subject incidence was analyzed using logistic regression with country and first child status as independent variables in the model. In both the binary and continuous analyses, pairwise comparisons between countries were conducted using Holm's procedure to adjust for the multiplicity of comparisons. Each specific episode of ANAP usage was classified by concurrent fever (yes/no) or infection (yes/no). Episodes were categorized as associated with Fever, Infection, both Fever and Infection, or neither fever nor infection. For each categorization, a generalized estimating equation (GEE) was used for analysis with country and first child as independent variables in the model. An ignorable working matrix was assumed for the GEE analysis with the empirical sandwich estimate used for the standard errors. Pair-wise comparisons across countries were conducted using Holm's procedure from the GEE analyses. Analyses on the episode level excluded subjects who reported no episodes.

Table 1 Covariates included in the Cox proportionate hazards analysis of time to persistent confirmed autoantibody positivity

Fixed Covariates		Hazard Ratio (95% CI)	Wald test p-value[a]
First-Degree Relative (Ref = No)		2.51 (2.06, 3.30)	<0.001
HLA (Ref = DR3/DR4)			
	DR4/DR4	0.69 (0.54, 0.88)	0.003
	DR4/DR8	0.70 (0.54, 0.91)	0.008
	DR3/DR3	0.46 (0.35, 0.60)	<0.001
	All Others	0.46 (0.29, 0.72)	<0.001
Gender (Ref = male)		0.77 (0.65, 0.92)	0.003
SNP			
	RS1004446_a	0.84 (0.74, 0.96)	0.010
	RS10517086_a	1.14 (1.00, 1.31)	0.050
	RS12708716_g	0.87 (0.76, 0.99)	0.034
	RS2292239_a	1.24 (1.09, 1.41)	<0.001
	RS2476601_a	1.55 (1.31, 1.83)	<0.001
	RS2816316_c	1.07 (0.91, 1.25)	0.429
	RS3184504_a	1.33 (1.17, 1.50)	<0.001
	RS4948088_a	0.74 (0.53, 1.04)	0.086
Ever Breastfed (Ref = No)		1.96 (1.01, 3.81)	0.042
Probiotics <3 Mo Age (Ref = No)		0.72 (0.55, 0.94)	0.015
Time Dependent Covariates			
Cumulative Number of Infections		1.02 (0.99, 1.03)	0.407
Cumulative Weeks Analgesic Use		1.02 (0.99, 1.04)	0.269

Number of persistent confirmed cases = 511
[a]Ho: Hazard Ratio = 1

Statistical analysis was performed using SAS version 9.3 (SAS Institute Inc., Cary, NC, U.S.A).

Results

Use of ANAP below the age of 2.5 years

The use of both acetaminophen and NSAIDs were very common in the study population. In the total cohort, 87.8% of children reported the use of acetaminophen and 45.4% of NSAIDs before the age of 2.5 years. The mean number of treatment episodes per year was 3.6 ± 2.1 and mean duration of treatment 8.5 ± 10.8 days per year in the total cohort (Fig. 1a–c).

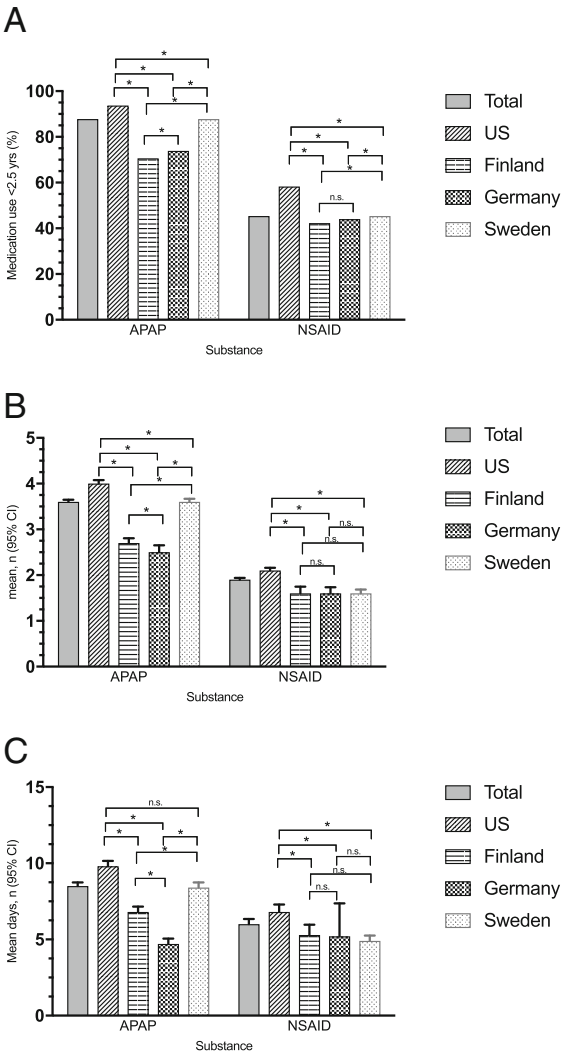

Fig. 1 Use of ANAP below 2,5 years of age. *:$p < 0.05$, n.s.: non significant. All significances corrected for multiple comparisons using Holm procedure. **a** Prevalence of analgesic/antipyretic use. **b** Treatment episodes per year. **c** Duration of medication use per year

Acetaminophen use

Swedish parents reported a significantly higher prevalence of acetaminophen use (94.5%), followed by U.S. (93.7%), Finnish (73.9%), and German parents (70.1%). Prevalence differed between all countries (Finland vs. Germany: $p = 0.035$, all other $p < 0.001$). U.S. parents reported the highest number of treatment episodes per year and highest total duration of treatment per year (mean 4.0 ± 2.3 episodes; mean 9.8 ± 13.5 days), followed by Swedish (mean 3.6 ± 2.1 episodes; mean 8.4 ± 8.6 days), Finnish (mean 2.7 ± 1.9 episodes; mean 6.8 ± 6.4 days), and German parents (mean 2.5 ± 1.6 episodes; mean 4.7 ± 3.7 days). All country differences, according to number of treatment episodes, were statistically significant (Finland vs. Germany: $p = 0.014$, all others $p < 0.001$) (Fig. 1). Children born as the first child in the family had more often been given acetaminophen during their first 2.5 years of life compared to children with older siblings (OR 1.26; 95% CI 1.07, 1.49; $p = 0.007$). The number of episodes of treatment with acetaminophen was also higher (difference in least square means 0.15; 95% CI 0.05, 9.24; $p = 0.003$). No difference could be seen regarding the number of days treated (difference in least square means 0.11; 95% CI -0.38, 0.60; $p = 0.111$).

NSAID use

The highest prevalence of NSAID use was reported by U.S. parents (58.3%), followed by German (44.1%), Finnish (42.3%), and Swedish parents (29.0%). All country differences, except between Finland and Germany, were statistically significant (Finland vs. Germany: $p = 0.177$, all others $p < 0.001$). U.S. parents reported the highest number of treatment episodes with NSAID per year (mean 2.1 ± 1.4 episodes; mean 6.8 ± 11.2 days), followed by Swedish, Finnish, and German parents (mean 1.6 ± 1.2; mean 1.6 ± 1.2; mean 1.6 ± 1.1) Total duration of treatment was highest in the U.S. (mean 6.8 ± 11.2 days), followed by Finland (mean 5.3 ± 9.3 days), Germany (mean 5.2 ± 17.2 days), and Sweden (mean 4.9 ± 4.8 days). Both the mean number of treatment episodes and mean total duration of treatment were significantly higher in the U.S. compared to the other countries ($p < 0.001$). No other significant country differences could be seen (Fig. 1). The prevalence of NSAID use during the first 2.5 years of life were lower in first-born children (OR 0.86; 95% CI 0.78, 0.95; $p = 0.002$) and they were also treated fewer times (difference in least square means −0.14; 95% CI -0.22, −0.05; $p = 0.001$). No differences could be seen regarding the number of days treated (difference in least square means −0.22; 95% CI -0.92, 0.48; $p = 0.143$).

Differences in use for febrile/infectious episodes and noninfectious use

In the total cohort, 74.1% of acetaminophen use and 82.0% of NSAID use was given in conjunction with either fever, infection or both, with 43.8% acetaminophen use and 51.0% NSAID episodes being combined fever and infection. U.S. parents reported a significantly higher proportion of doses given without fever or infection for both acetaminophen (40.4%) and NSAID (26.3%) compared to the other three countries ($p < 0.001$). Acetaminophen use in feverish infectious episodes had the highest proportion among German and Swedish children (68.5% and 63.2%;), followed by Finland with 57.9% and the U.S. with 23.5% (all p-values for differences between countries were $p < 0.001$, except between Germany and Swedenwas $p = 0.003$).

For NSAID use without fever or infection, the U.S. parents reported the highest proportion (26.3%), followed by Finland (7.7%), Germany (5.9%), and Sweden (3.7%) (difference between Finland and Sweden $p = 0.006$; between Germany and Sweden $p = 0.01$; all others $p < 0.001$) (Fig. 2, Table 2).

Islet autoimmunity

Hazard ratios for islet autoimmunity were estimated for cumulative use of acetaminophen and NSAID with or without concomitant fever and for a joint variable of cumulative total ANAP use with or without fever. A significant hazard was only found for use of acetaminophen in the presence of fever for islet autoimmunity at age 3 years (HR 1.05; 95% CI 1.01-1.09; $p = 0.024$). The hazard was not significant for islet autoimmunity at 6 years of age ($p = 0.193$).

Separate analysis of exposure before 90, 180 and 365 days of life found a significant hazard for seroconversion at age 3 years (HR 1.06; 95% CI 1.00-1.12; $p = 0.011$) for use of acetaminophen with concurrent fever before 1 year of age, but not before 90 or 180 days of life ($p = 0.91$ and $p = 0.54$, respectively). No other significant hazards could be seen for treatment with acetaminophen or NSAID in the presence or absence of fever (Table 3).

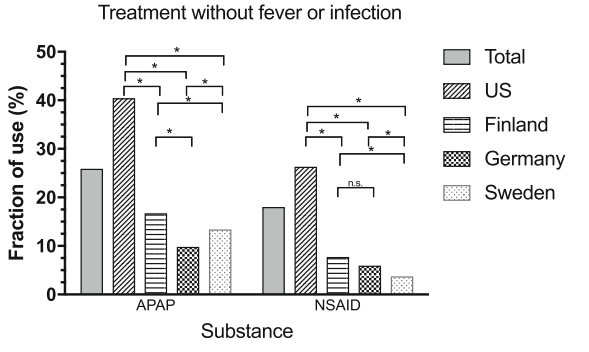

Fig. 2 Fraction of treatment without fever and infection. *:$p < 0.05$, n.s.: non-significant. All significances corrected for multiple comparisons using Holm procedure

Discussion

In this study, we investigated the use of ANAP in children below the age of 2.5 years, and the impact of such use on the development of islet autoimmunity before 6 years of age in the large, longitudinal, international TEDDY cohort.

The widespread use of acetaminophen among young children has been of interest due not only to possible side effects, but also possible immunological effects. Several papers have investigated the impact on immune response and the development of autoimmunity [11, 12, 32, 33]. The data on childhood asthma is conflicting, with some studies showing an increased risk and others showing none [14, 15, 34]. The use of prophylactic acetaminophen in conjunction with childhood vaccinations has also shown possible effects on antibody responses [1, 7, 35]. In this study, we found a significant but weak increased hazard ratio associated with the use of acetaminophen and concomitant fever before the age of 2.5 years and persistent confirmed islet autoimmunity at age 3 years. However, this effect was not seen with islet autoimmunity at age 6 years or if acetaminophen was used for other reasons. It is therefore unlikely, although possible, that this is a true effect. The type of infection causing the fever may be a confounding factor. No such effect was seen with the use of NSAIDs or the combination of acetaminophen and NSAIDs, either when given with or without fever.

The use of acetaminophen and NSAIDs for the treatment of children has been previously described from a medical standpoint [36, 37]. On the other hand, very little has been described regarding practical use in the pediatric population. This analysis within a large international cohort provides some of the first data regarding pediatric use of ANAP. As expected,the majority of treatment episodes for this young cohort were in conjunction with fever and/or infection. It is worth mentioning that there are significant differences between the TEDDY countries regarding the use of both acetaminophen and NSAIDs. The U.S. stands out for both greater prevalence of use and greater number of episodes of treatment per year, followed closely by Sweden in regards to acetaminophen use. U.S. parents were also just as likely to report using these medications during episodes associated with infection than non-infectious episodes. Additionally, they were more likely to use ANAP when there was no associated fever. It may be a common practice of American physicians to prescribe a

Table 2 Summary of treatment episodes associated with fever and/or infection

			US	Finland	Germany	Sweden	Total Cohort	Country differences					
								U-F	U-G	U-S	F-G	F-S	G-S
Total episodes		n	25,340	7427	2091	17,285	52,643						
Acetaminophen	Fever and infection	n	4272	2907	983	9224	17,386	<0.001	<0.001	<0.001	<0.001	<0.001	0.003
	Fever or infection		6557	1277	311	3421	12,066						
	No fever and no infection	n	7348	841	141	1953	10,283	<0.001	<0.001	<0.001	<0.001	0.006	0.01
NSAID	Fever and infection	n	2680	1273	477	1572	6002	<0.001	<0.001	<0.001	<0.001	<0.001	0.024
	Fever or infection	n	2472	717	125	330	3644						
	No fever and no infection	n	1841	167	38	74	2120	<0.001	<0.001	<0.001	0.174	<0.001	0.041

Country differences: U = US, G = Germany, F = Finland, S = Sweden, Country differences described as *p*-value for difference between the respective countries

combination therapy approach to the use of analgesics and antipyretics forthe sustained management of fever. However, parents may then assume that even prophylactic use should be a combination therapy.

We also found that first-born children were preferentially given acetaminophen, both in prevalence of use and in the higher number of treatment episodes than for their younger siblings. The inverse relationship was observed for NSAID use, in which both the prevalence and number of treatment episodes were lower for first-born children. We can only speculate on the possible rationale behind this finding since, to our knowledge, no earlier study has presented similar data. It is possible that acetaminophen is perceived by first-time parents as a better tolerated treatmentthan NSAIDS, a perception that fades by the time younger siblings require treatment.

Country-specific differences in the use of analgesics may be culturally influenced. The lower incidence of use of all standard analgesics in Germany could reflect the prevalence of Complimentary Alternative Medicine (CAM) in this country. According

Table 3 Hazard ratios for seroconversion to persistent islet autoimmunity at 3 and 6 years of age for analgesic variables of interest

Analgesic Variable	Exposed subjects n (%)	3 Year Analysis 398 antibody + subjects HR (95% CI) *p*-value	6 Year Analysis 511 antibody + subjects HR (95% CI) *p*-value
Acetaminophen, any exposure	7496 (87.8%)	1.01 (0.97, 1.05) 0.603	1.01 (0.98, 1.05) 0.576
Acetaminophen with fever + infection	5179 (60.6%)	1.05 (1.01, 1.09) 0.022	1.03 (0.99, 1.08) 0.189
Exposed <90 days of life	220 (2.6%)	0.97 (0.59, 1.61) 0.914	1.00 (0.68, 1.46) 0.986
Exposed <180 days of life	1519 (17.8%)	1.07 (0.87, 1.32) 0.542	1.06 (0.88, 1.27) 0.527
Exposed <365 days of life	3795 (44.4%)	1.06 (1.00, 1.12) 0.011	1.05 (0.99, 1.11) 0.101
Acetaminophen without fever or infection	5941 (69.6%)	1.02 (0.98, 1.06) 0.346	1.01 (0.96, 1.05) 0.769
Exposed <90 days of life	4016 (47.0%)	1.00 (0.90, 1.11) 0.994	0.99 (0.85, 1.14) 0.837
Exposed <180 days of life	4597 (53.8%)	0.98 (0.84, 1.14) 0.814	0.97 (0.84, 1.13) 0.729
Exposed <365 days of life	5310 (62.2%)	1.03 (0.99, 1.06) 0.114	1.02 (0.99, 1.06) 0.228
NSAID, any exposure	3874 (45.4%)	1.01 (0.97, 1.05) 0.753	1.01 (0.97, 1.04) 0.763
NSAID with fever + infection	2652 (31.0%)	1.01 (0.97, 1.05) 0.673	1.01 (0.98, 1.05) 0.459
Exposed <90 days of life	22 (0.3%)	1.01 (0.92, 1.10) 0.897	1.00 (0.92, 1.09) 0.960
Exposed <180 days of life	223 (2.6%)	0.99 (0.88, 1.11) 0.822	1.00 (0.94, 1.07) 0.927
Exposed <365 days of life	1318 (15.4%)	1.01 (0.97, 1.06) 0.530	1.02 (0.98, 1.05) 0.378
NSAID without fever or infection	1955 (22.9%)	1.00 (0.96, 1.05) 0.856	1.01 (0.97, 1.05) 0.623
Exposed <90 days of life	222 (2.6%)	1.00 (0.88, 1.12) 0.943	0.99 (0.88, 1.12) 0.874
Exposed <180 days of life	458 (5.4%)	0.99 (0.88, 1.11) 0.815	1.00 (0.94, 1.07) 0.956
Exposed <365 days of life	1120 (13.1%)	1.01 (0.96, 1.06) 0.824	1.01 (0.97, 1.05) 0.638
Any analgesic, any exposure	7744 (91%)	1.02 (0.99, 1.05) 0.130	1.02 (0.99, 1.04) 0.267
Any analgesic with fever + infection	5699 (67%)	1.06 (0.97, 1.15) 0.219	1.02 (0.94, 1.10) 0.667

Each hazard ratio is calculated from a Cox proportional hazards model with the analgesic variable and covariates indicated in text

to a cross-sectional survey of German physicians in 2007, more than two-thirds of patients in Germany use CAM provided either by physicians or non-medical practitioners ("Heilpraktiker") [38]. In 2007, only 40% of adults in the U.S. had used CAM therapy in the past 12 months. Children in the U.S. whose parents used CAM were almost five times as likely (23.9%) to use CAM than children whose parent did not use CAM (5.1%) [39]. The reasons underlying greater use of acetaminophen among Swedish parents is more unclear but may be the result of acetaminophen being widely available and perceived as safe and effective.

The TEDDY study is one of the largest longitudinal pediatric cohorts studied. The data analyzed herein has been collected from parent reports given in writing and after discussion with a TEDDY nurse. For participating children, missing data is uncommon. Follow-up is continuous from age 3 months which minimizes recall bias. The possibility to adjust for confounding factors in the statistical analysis is great due to the availability of comprehensive data on the child's living conditions. All previously described risk factors for T1D and islet autoimmunity are also entered into the statistical analysis of the effect of analgesics on islet autoimmunity.

The limitations of this study include our reliance on parent-reported symptoms and dosages of ANAP. The size of the cohort also makes it challenging to confirm diagnoses and treatment plans via patient records. Notably, most of the reported infections were presumed to be viral infections for which no medical advice had been sought. In addition, the widespread use of ANAP in this age group poses a significant statistical problem since the exposed group widely surpasses the non-exposed group. Even with the large sample size, the resulting correlation between IA and acetaminophen in combination with fever must therefore be interpreted with caution.

Conclusions

In conclusion, the use of ANAP to treat fever and infection is widespread in the TEDDY cohort but shows significant differences depending on study site. The prevalence of use of both acetaminophen and NSAIDs are highest in the U.S. and lowest in Finland and Germany. Use of both NSAIDs and acetaminophen for non-infectious purposes are significantly more common among children in the U.S. compared to those in Europe. No convincing effect on risk for autoimmunity can be seen in the analysis except for a small effect by acetaminophen in combination with fever, and then only for autoimmunity at 3 years of age.

Additional files

Additional file 1: Ethical review boards and Committees granting ethical approval to the TEDDY study. Listing of all ethical review boards and committes that has granted approval to the TEDDY study for the respective sites. (DOCX 59 kb)

Additional file 2: Characteristics of the subjects used in analysis for analgesic use and islet cell autoimmunity. A table the prevalence of covariates in the present analysis including HLA-DQ genotype, Gender, first degree relative, breastfeeding, probiotic use and presence of included single nucleotide polymorphisms. (DOCX 106 kb)

Additional file 3: List of Drugs defined as analgesics used in first 2.5 years in the TEDDY study. List of all included drugs, recorded to having been given to TEDDY children before age 2.5 years and classified as analgesics. Drugs classified as NSAID's are marked with a star (*). (DOCX 30 kb)

Additional file 4: List of all ICD-10 codes, recorded among TEDDY children before age 2.5 years, and classified as an infection. (DOCX 72 kb)

Abbreviations

ANAP: Analgesic-Antipyretic; CAM: Complimentary alternative medicine; GEE: Generalized estimating eq.; IA: Islet Autoimmunity; NSAID: Non-steroidal anti-inflammatory drugs; T1D: Type 1 Diabetes; TEDDY: The environmental determinants of diabetes in the young study

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Availability of data and materials
The data that support the findings of this study are available from the NIDDK repository (http://niddkrepository.org) approximately 6 months after the manuscript has appeared in print. Access for the data submitted to the NIDDK Data Repository will be determined by the NIDDK. All investigators who receive TEDDY resources must agree to acknowledge the TEDDY Study and the NIDDK central repository. This approach is fully compliant with the NIH public data sharing policy (http://grants.nih.gov/grants/policy/data_sharing).

Authors' contributions
ML researched the data and wrote the manuscript. LJS was involved in the drafting of the manuscript and reviewed/edited the manuscript. RT made the statistical analyses, researched data and reviewed/edited the manuscript. MH, HEL, BJ, PG, CC, MS, GH researched data and reviewed/edited the manuscript. WH, AGZ, MR, ÅL, JT, JS, BA and JK designed the study, researched data, and reviewed/edited the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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Paper IV

Pediatric Diabetes

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Pediatric Diabetes

Original Article

Reduced morbidity at diagnosis and improved glycemic control in children previously enrolled in DiPiS follow-up

Lundgren M, Sahlin Å, Svensson C, Carlsson A, Cedervall E, Jönsson B, Jönsson I, Larsson K, Lernmark Å, Neiderud J, Vigård T, Elding Larsson H, the DiPiS study group. Reduced morbidity at diagnosis and improved glycemic control in children previously enrolled in DiPiS follow-up. Pediatric Diabetes 2014.

Aims/hypothesis: Children participating in longitudinal type 1 diabetes prediction studies were reported to have less severe disease at diabetes diagnosis. Our aim was to investigate children who from birth participated in the Diabetes Prediction in Skåne (DiPiS) study for metabolic status at diagnosis and then continued to be followed for 2 yr of regular clinical care.
Methods: Children, followed in DiPiS before diagnosis, were compared to children in the same birth cohort, who did not participate in follow-up. Metabolic status, symptoms at diagnosis as well as hemoglobin A1c (HbA1c) and doses of insulin at 3, 6, 12, and 24 months after diagnosis were compared.
Results: Children, followed in DiPiS and diagnosed at 2–12 yr of age, had 0.8% (9 mmol/mol) lower HbA1c at diagnosis than those who were not followed ($p = 0.006$). At diagnosis, fewer DiPiS children had symptoms ($p = 0.014$) and ketoacidosis at diagnosis were reduced (2% compared to 18%, $p = 0.005$). During regular clinical care, HbA1c levels for the DiPiS children remained lower both at 12 (0.4% (4 mmol/mol); $p = 0.009$) and 24 months (0.8% (9 mmol/mol) $p < 0.001$) after diagnosis, despite no difference in total daily insulin between the two groups.
Conclusions: Participation in prospective follow-up before diagnosis of type 1 diabetes leads to earlier diagnosis with fewer symptoms, decreased incidence of ketoacidosis as well as better metabolic control up to 2 yr after diagnosis. Our data indicate that metabolic control at the time of diabetes diagnosis is important for early metabolic control possibly affecting the risk of long-term complications.

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Key words: diabetic ketoacidosis – follow-up studies – HbA1c – type 1 diabetes

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The triggering event for islet autoimmunity eventually resulting in the clinical onset of type 1 diabetes, is still unknown. This is true for both the initial insult of the autoimmune process but also for factors governing the time it takes for autoimmunity to lead to clinical disease. Prediction of who will develop the disease and when is critical for attempts to stall and perhaps to stop the disease process. The human leukocyte antigen (HLA) DQ locus is presumed to account for 50% of the genetic risk of type 1 diabetes (1). More than 40 additional non-HLA risk genes have been identified in recent genome-wide association studies (2). Islet autoantibodies are used to estimate the risk for diabetes and it is well-established that the risk is increasing with an increasing number of autoantibodies (3).

Children born with increased genetic risk for type 1 diabetes have been followed in several prospective longitudinal studies. The focus has been the development and maintenance of the beta-cell autoimmune process. Only a few studies have been published on the status of the participants both at the time of diagnosis of diabetes and during follow-up after diagnosis. Participants in longitudinal studies have been shown to have fewer metabolic abnormalities at diagnosis and a lower frequency of diabetic ketoacidosis (4–6) as well as having a milder clinical course in the first year after diagnosis (7). This may be due to intense follow-up with measures of plasma glucose, hemoglobin A1c (HbA1c) or glucose tolerance tests. However, the parent's knowledge of the risk of disease may also result in an earlier diagnosis through increased vigilance regarding symptoms.

DiPiS is a prospective study on type 1 diabetes prediction in Sweden. The aim of this study was to investigate the disease at diagnosis and the metabolic control during 2 yr of regular clinical care after diagnosis, after participation in the DiPiS study (8). We compared the DiPiS children with children born during the same years, who developed diabetes outside of the DiPiS study.

Methods

Participants

In the Diabetes Prediction in Skåne (DiPiS) study, 2500 children with genetic risk for type 1 diabetes in southern Sweden are prospectively studied in a 15-yr longitudinal investigation (9). Between September 2000 and August 2004 approximately 48 000 children were born in the five participating hospitals and after oral consent from the mother, 35 683 of those children had umbilical cord blood samples collected, for HLA genotyping (Fig. 1). When the child was 2 months old, the parents were invited to participate in DiPiS by giving written consent and answering questionnaires regarding, among other things, pregnancy, socioeconomic factors, birth weight and length, hereditary factors and stressors during pregnancy. A total of 25 378 questionnaires (71%) were returned by the parents of the study participants of which 92% wanted to participate.

A risk score was developed based on HLA risk genotype for type 1 diabetes, presence of maternal infections during pregnancy, maternal diabetes, cord blood autoantibodies and high or low relative birth weight. On the basis of this risk score, approximately 6000 children who had answered the 2 months questionnaire were selected for annual follow-up. Only parents of the 3680 children who returned the 2-yr questionnaire received HLA-based risk information. Participants are screened yearly, from the age of 2 yr, with a questionnaire and blood sampling for islet autoantibody analysis. Those children who develop two or more autoantibodies are offered follow-up every 3 months by a pediatrician, with autoantibody sampling, random plasma glucose, HbA1c, growth parameters, questionnaire and oral glucose tolerance test (OGTT) annually.

Up to July 2013, a total of 143 of the children with cord blood samples taken at birth had developed type 1 diabetes. Of those, 14 children developed diabetes before 2 yr of age and were therefore not given risk information or the possibility to participate in the follow-up. After exclusion of children who had developed other types of diabetes and those diagnosed before the age of 2, 129 children remained in the following three subgroups (Fig. 1):

Group A: DiPiS children participating in intense follow-up. This group is comprised of children who had developed multiple autoantibodies and have participated in follow-up every 3 months (n = 33).

Group B: DiPiS children who participated in annual autoantibody sampling and questionnaires. This group has answered the 2-yr questionnaire, have been invited to the DiPiS follow-up and have received information about the risk for type 1 diabetes (n = 18).

Group C: Children with umbilical cord samples but who declined participation in follow-up and risk information or where not invited to the study due to low HLA risk for type 1 diabetes. None of these

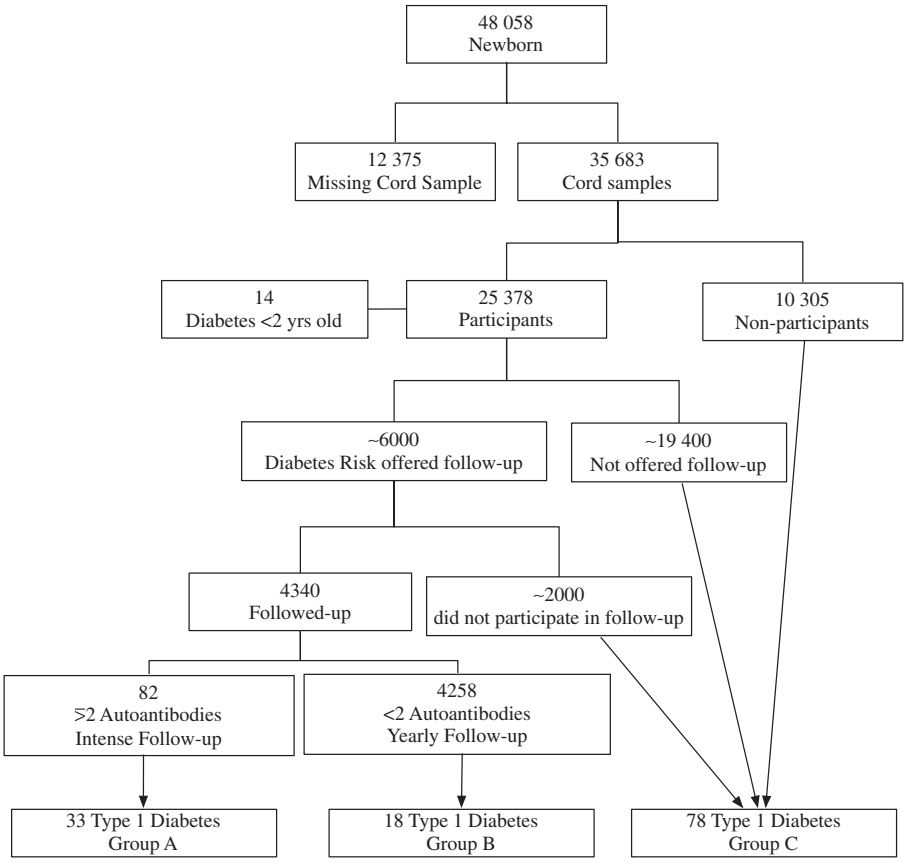

Fig. 1. Study design and selection of participants.

children received information about type 1 diabetes risk (n = 78).

The analysis in this study was made as a comparison between children in groups A and B together [Follow-Up (FU) group] with group C [No Follow-Up group (NFU)], who were not involved in any kind of follow-up.

Compliance to follow-up in group A was good as only 15% (n = 12) of the autoantibody positive subjects have dropped out. The dropout rate is variable, as some of the dropout children have opted to return to a yearly follow-up. Group B has 2160 subjects in active follow-up out of the original 4258, representing a dropout rate of 49%. Groups A and B children who stopped follow-up have not been included in the NFU group and most importantly none of the children in the NFU group have received any information on HLA-DQ-related risk for type 1 diabetes. Group A children, who switched to annual follow-up has been included in group B.

A total of 32 participants have not reached 24 months of clinical follow-up at the present time: 8 in group A, 6 in group B, and 18 in group C. The exact number of samples available for analysis are indicated in Fig. 2.

Definition of DKA

Diabetic ketoacidosis was defined as a blood pH < 7.3 and severe diabetic ketoacidosis as blood pH < 7.1 at diagnosis. Samples were drawn at diagnosis in an emergency room setting.

HLA DQ typing

HLA DQ genotyping was performed on cord blood with allele-specific HLA DQ A1 and B1 probes as described elsewhere (9). HLA was classified as HLA-DQA1*0501-DQB1*0201 (DQ2) or HLA-DQA*0301-DQB1*0302 (DQ8) and stratified into four risk groups: DQ 2/8, DQ8/8 or 8/X, DQ2/2, or 2/X or DQ X/X (X is neither DQ2 nor DQ8).

Autoantibody analysis

Follow-up samples and samples from diagnosis of diabetes were analyzed for autoantibodies to GAD65 and IA-2 with radio immune assays described elsewhere (10) and with a commercially available ELISA kit, according to the manufacturers instructions (RSR Limited, Cardiff, UK). Samples from follow-up

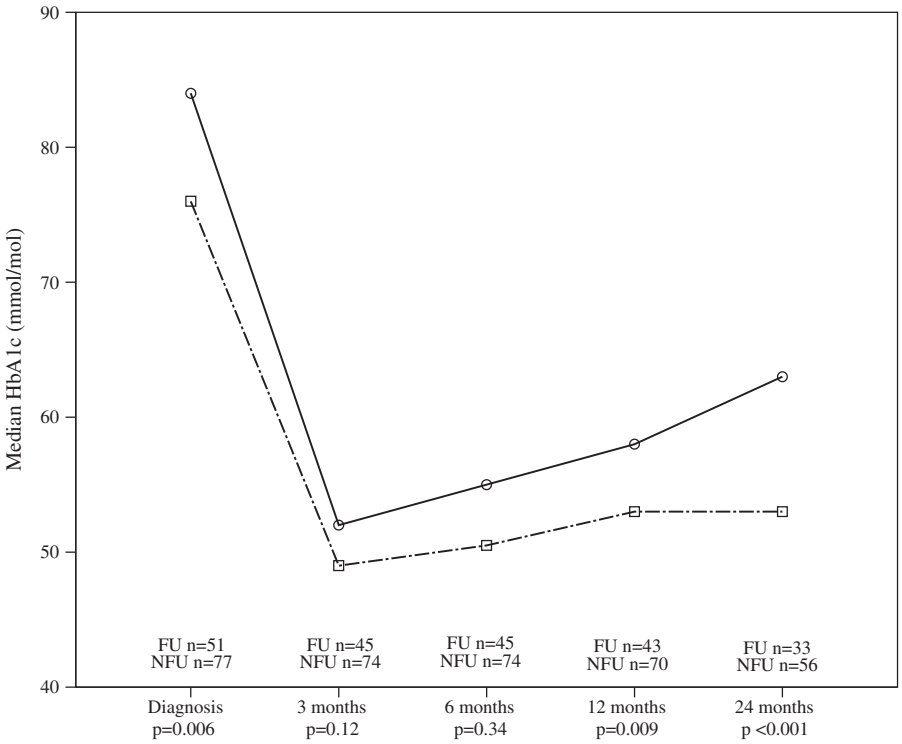

Fig. 2. HbA1c levels at diagnosis and the first 2 yr after diagnosis in the FU and NFU groups. Solid line, no follow-up group; Dotted line, follow-up group.

visits and diabetes diagnosis were additionally analyzed for autoantibodies to insulin (IAA) and radio immune assay of the three amino acid variants of zinc transporter 8 (ZnT8RA, arginine 325 zinc transporter 8 autoantibody; ZnT8WA, tryptophan 325 zinc transporter 8 autoantibody; ZnT8QA, glutamine 325 zinc transporter 8 autoantibody) autoantibodies. For IAA performed before 2011 and all ZnT8 autoantibodies the method is described elsewhere (11). For IAA samples analyzed after 2010, the following alteration to the assay has been made: to the buffer used for incubation [TRIS-buffer (pH 8.0) with 1% (v/v) Tween 20] 1% w/v bovine serum albumin was added to prevent non-specific binding. In the competitive assay, instead of arbitrary units, U/mL has been calculated using a standard curve. The standard curve represented seven different concentrations (3–358 U/mL) and the concentration was plotted against cpm values on a Log2 scale.

Data collection

Pediatricians at the pediatric clinics in the Region Skåne continuously reported children from the DiPiS cohort when they developed diabetes. Additional information was gained from on-going studies, including all children diagnosed in Skåne (the Skåne study) and Sweden (the Better Diabetes Diagnosis study) (12). Data regarding metabolic parameters, symptoms at diagnosis and clinical post diagnosis follow-up data on HbA1c and insulin doses were retrieved from individual electronic patient records (Melior, Siemens AG, Berlin, Germany). Insulin doses were analyzed as total units of insulin per kg and day. Follow-up data was recorded after diagnosis at 3, 6, 12, and 24 ± 1 month. The clinical care of the diagnosed patients is ongoing, and some children with type 1 diabetes have therefore yet to reach 24 months after diagnosis.

Statistical methods

Data were analyzed using SPSS version 21 (Spss inc, Chicago, IL, USA). Differences in categorical variables were tested using Pearson's chi-squared test or Fisher's exact test when appropriate. Mann–Whitney *U* test was used for continuous variables. Correlation analysis between HbA1c and insulin doses was performed using Spearman's rho. All HbA1c analyses were performed using IFCC (mmol/mol) and results were then recalculated to NGSP (%). Missing data was excluded on a variable-by-variable basis to maximize use of the data. Bonferroni correction for multiple comparisons was performed where applicable. P-values < 0.05 were considered statistically significant.

The Regional Ethics Review Board in Lund, Sweden, approved the study.

Results

Baseline characteristics

The baseline characteristics of the 129 DiPiS children diagnosed with type 1 diabetes are summarized in Table 1. The FU/NFU groups did not differ in gender ($p = 0.38$) or mean age at diagnosis of diabetes ($p = 0.45$). Two children were negative for islet autoantibodies at diagnosis of diabetes (Table 1). One was antibody positive in control samples 1 yr after diagnosis and the other had a clinically typical type 1 diabetes. The HLA risk genotypes DQ 2/8, DQ 8/8, DQ 8/X, DQ 2/2, and DQ 2/X were found in 97% in the children with intense follow-up (group A), 100% in the group with some follow-up (group B), and 86% in the group (group C) without follow-up (Table 1).

Clinical characteristics at diagnosis

The presence of the following symptoms was recorded at diagnosis: polyuria, polydipsia, weight loss, and ketoacidosis. The presence of at least one symptom was less in the FU (84%) compared to the NFU group [97%; OR 0.14 (95% CI = 0.03–0.70; $p = 0.014$), Bonferroni corrected p-value 0.028; Table 2].

Diabetic ketoacidosis

The proportion of children presenting with diabetic ketoacidosis ($pH < 7.3$) was lower in the FU (2%) compared to the NFU group [18%; OR 0.091 (95% CI = 0.01–0.80; $p = 0.005$) Bonferroni corrected p-value 0.010). Regarding severe ketoacidosis ($pH < 7.1$) no significant differences could be seen with an incidence of 2% in the FU and 5% in the NFU group. None of the patients enrolled in intense follow-up presented with diabetic ketoacidosis.

Hemoglobin A1c (HbA1c)

At the time of diabetes diagnosis, the FU group had a lower median HbA1c, 9.2% (77 mmol/mol) compared to 10.0% (87 mmol/mol) in the NFU group ($p = 0.006$) (Table 2). At 3 and 6 months after diagnosis no significant differences were observed. At 12 months after diagnosis, the children in the FU group had lower HbA1c, 7.0% (53 mmol/mol) compared to 7.4% (57 mmol/mol) in the NFU group ($p = 0.009$) (Table 2, Fig. 2). At 24 months after diagnosis, this difference increased with the FU group having median HbA1c 7.0% (53 mmol/mol) compared to 7.8% (62 mmol/mol) in the NFU group ($p < 0.001$). All differences remained statistically significant after correction for multiple comparisons (Table 3, Fig. 2). In the total cohort at diagnosis, patients, with ketoacidosis (11.5% (102 mmol/mol) had a higher median HbA1c than patients without [9.6% (81 mmol/mol); $p < 0.001$]. However, HbA1c did not differ between these two groups during the first 2 yr after diagnosis (3 months $p = 0.386$; 6 months $p = 0.080$; 12 months $p = 0.126$; 24 months $p = 0.793$).

Insulin dose and remission

Three months after diagnosis, children in the FU group had a lower total daily dose of insulin (0.47 U kg^{-1} d^{-1}) compared to the NFU group (0.52 U kg^{-1} d^{-1}; $p = 0.026$), while no differences were observed at 6, 12,

Table 1. Baseline characteristics of the DiPiS study groups at diagnosis

		Study group in DiPiS		
		A	B	C
n		33	18	78
Age at diagnosis (yr)	Mean ± SD	6.5 ± 2.6	7.3 ± 3.2	6.5 ± 2.6
Gender (n)	Female	20 (61%)	9 (50%)	38 (49%)
	Male	13 (39%)	9 (50%)	40 (51%)
HLA-DQ (n)	2/8	15 (46%)	6 (33%)	34 (44%)
	8/8, 8/X (X is not 2)	14 (42%)	9 (50%)	18 (23%)
	2/2, 2/X (X is not 8)	3 (9%)	3 (17%)	15 (19%)
	X/X (X is not 2 or 8)	1 (3%)	0	11 (14%)
Autoantibody pos. (n)	GAD65A	24 (73%)	13 (72%)	52 (67%)
	IAA-A	11 (33%)	8 (44%)	33 (42%)
	IA2-A	28 (85%)	13 (72%)	51 (65%)
	ZnT8RA	17 (52%)	8 (44%)	36 (46%)
	ZnT8WA	18 (55%)	8 (44%)	29 (37%)
	ZnT8QA	12 (36%)	5 (28%)	18 (23%)
Antibodycount (n)	Negative	0	0	2 (3%)
	Single	4 (12%)	4 (22%)	12 (15%)
	Multiple	27 (82%)	14 (78%)	60 (77%)
	Missing	2 (6%)	0	4 (5%)

Table 2. Incidence of diabetes related symptoms at diagnosis in the FU and NFU group

	n	Follow-up Incidence (%)	No follow-up Incidence (%)	p*	p†	OR	95% CI
Symptoms	129	84	97	0.014	0.028	0.141	0.029–0.696
Polydipsia	129	80	96	0.006	0.018	0.16	0.043–0.63
Polyuria	129	76	96	0.001	0.003	0.13	0.035–0.49
Weight loss	122	46	65	0.034	0.10	0.45	0.22–0.95
DKA	129	2	18	0.005	0.01	0.091	0.012–0.72
Severe DKA	129	2	5	ns	ns	0.50	0.051–4.94

DKA, diabetic ketoacidosis (pH $<$ 7.3); Severe DKA, diabetic ketoacidosis (pH $<$ 7.1).
*p-Value of Mann–Whitney *U* test.
†Bonferroni corrected p-value.

Table 3. HbA1c at onset and HbA1c and total daily dose of insulin for the 2 yr following diabetes diagnosis in the FU and NFU groups

		n	Follow-Up Median (IQR)	No Follow-Up Median (IQR)	p*	p†
HbA1c at diagnosis	% (mmol/mol)	128	9.2 (8.0–10.5) (77 (64–91))	10.0 (9.0–11.2) (86 (75–99))	0.006	0.03
HbA1c, 3 months	% (mmol/mol)	119	6.5 (6.1–7.0) (48 (43–53))	6.7 (6.2–7.5) (50 (44–58))	0.120	0.60
HbA1c, 6 months	% (mmol/mol)	119	6.6 (6.0–7.5) (49 (43–59))	7.1 (6.2–7.6) (54 (44–60))	0.344	>1
HbA1c, 12 months	% (mmol/mol)	113	7.0 (6.5–7.4) (53 (48–57))	7.4 (6.5–7.4) (57 (51–65))	0.009	0.045
HbA1c, 24 months	% (mmol/mol)	89	7.0 (6.5–7.7) (53 (48–61))	7.8 (7.1–8.4) (62 (54–69))	<0.001	<0.001
TDD, 3 months	U kg^{-1} d^{-1}	125	0.47 (0.30–0.58)	0.52 (0.39–0.65)	0.026	0.13
TDD, 6 months	U kg^{-1} d^{-1}	120	0.62 (0.41–0.76)	0.62 (0.41–0.76)	0.443	>1
TDD, 12 months	U kg^{-1} d^{-1}	113	0.75 (0.52–0.89)	0.75 (0.59–0.90)	0.511	>1
TDD, 24 months	U kg^{-1} d^{-1}	89	0.76 (0.63–0.93)	0.89 (0.63–0.93)	0.098	0.49

IQR, interquartile range; TDD, total daily dose of insulin.
*p-Value of Mann–Whitney *U* test.
†Bonferroni corrected p-value.

and 24 months after diagnosis (Table 3). No differences in total daily insulin dose (TDD) were observed after Bonferroni correction for multiple analyses. A weak but significant correlation between insulin dose and HbA1c was detected in the total study group 24 months post diagnosis ($\rho = 0.376$, $p < 0.001$). No differences in remission, defined as TDD $<$ 0.5 U kg^{-1} d^{-1}, were observed between the groups at any time.

Discussion

The main findings in this study is that children participating in a prospective long-term follow-up before the diagnosis of type 1 diabetes are healthier at diabetes diagnosis and have better metabolic control in standard clinical care up to 2 yr after diagnosis, compared to children who have not been followed in a study. Specifically, we found that children participating in follow-up more often presented without any reported diabetes-related symptoms. Children in follow-up (FU) therefore had decreased rates of polydipsia, polyuria, and weight loss, between 15 and 20% less, than the No follow-up (NFU) group. These findings are in line with earlier diabetes prediction studies such as DPT-1, DAISY, and the BABYDIAB (5, 7, 13). The TEDDY study described 11.3% ketoacidosis, which was lower than similar longitudinal studies or registries (6). It was therefore of interest that the NFU group in our study had similar rates of ketoacidosis at diagnosis compared to data from the Swedish pediatric diabetes registry from 2012 (14), whereas our FU group had only 2% ketoacidosis at diagnosis. This very low frequency of ketoacidosis is in part explained by the fact that our DiPiS children were not followed until they were 2 yr of age.

Reducing the incidence of ketoacidosis is important as it carries a risk of mortality as well as reduced beta-cell function (15–17). As DiPiS was designed to follow children and give risk information from 2 yr of age, children diagnosed with diabetes before this age (n = 14) were missed. It is known that children in the youngest age group have a higher frequency of diabetic ketoacidosis at diagnosis (16, 18). It cannot be excluded that our frequency of ketoacidosis in the

NFU group might have been higher if the DiPiS study design would have included children from an earlier age. It is possible that the younger age group may have had the greatest benefit of having parents, who were informed about the risk of diabetes in their child.

Our results implicate that children participating in follow-up before diagnosis of type 1 diabetes have better metabolic control up to 2 yr after diagnosis, measured as a lower HbA1c. As HbA1c is strongly associated to the risk of long-term diabetes complications (19, 20), this is an important finding not previously described for a timeframe as long as 2 yr after participating in a longitudinal follow-up study. Furthermore, our finding that HbA1c was improved in the FU group was unexpected. We can only speculate why the study subjects showed an improved metabolic control for up to 2 yr. One possibility is that the families were better prepared for type 1 diabetes treatment and may have informed themselves in anticipation of a diagnosis. Another possibility is that the early diagnosis resulted in fewer metabolic abnormalities. This may have led to a prolonged period of partial remission due to better beta-cell function at diagnosis. Previous studies have also addressed psychological problems related to early screening of infants and children, however, without finding serious adverse effects (21, 22). In this study, we show a beneficial effect to the participants with less severe status at diagnosis and an improved metabolic control up to 2 yr after diagnosis. These results may be important to the recruitment of study subjects for future follow-up studies.

A potential weakness of this study, was that the recording of data and the follow-up was not part of the original DiPiS protocol. For example, fasting or stimulated C-peptide at the time of diabetes diagnosis could not be done. During follow-up after diagnosis, c-peptide sampling was not a part of the regular clinical follow-up. However, no differences in remission, defined as TDD < 0.5 U kg^{-1} d^{-1}, could be seen between the FU and NFU groups. Also, the number of study participants with type 1 diabetes in this study may be regarded as rather small. This has to be taken into account when interpreting the data.

It could be argued that families willing to participate in the DiPiS study were more motivated, have a higher educational level or are more anxious about their child's health. Socioeconomic data is only available for the 25 0000 families, who filled out the 2-month questionnaire but not for the approximately 10 000 families who did not. Previous analyses in DiPiS have found that families were less likely to participate if the child was born in a hospital in a large city, the mother was either younger than 25 or older than 40 yr, was premature or twin. Mothers with diabetes were less likely to participate (4–6, 21). It is possible that other socioeconomic factors influenced the outcome after diagnosis, which was outside of the scope of this study.

This study represents a large part of the population as cord blood samples were obtained from 80% of the children born during 4 yr in the southernmost part of Sweden. The incidence of diabetes in the study cohort is 40/100 000 at the present time. The children diagnosed with diabetes are all cared for by public healthcare in six pediatric clinics and all physicians are using the same electronic patient records. It was therefore possible easily to access the patient charts. Differences in care are presumed to be small since all centers adhere to national guidelines on the management of pediatric type 1 diabetes and regular meetings are held in the region to ensure equal care.

In conclusion, our study shows that the DiPiS children who were enrolled at 2 yr of age in a longitudinal study on the prediction of diabetes were diagnosed at an early stage of the disease as HbA1c was lower and there were fewer symptoms including a lower frequency of ketoacidosis. In addition, the DiPiS children had better metabolic control after diagnosis when subjected to standard clinical care, demonstrating significantly lower HbA1c levels 12 and 24 months after diagnosis. Screening at birth for type 1 diabetes genetic risk and informing the parents may be sufficient to increase the awareness of diabetes symptoms to permit a diagnosis with less symptoms.

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Conflict of interest

The authors declare that there is no duality of interest associated with this manuscript.

Author Contributions

ML analyzed the data and wrote the manuscript. ÅS and CS were involved in early interpretation, collection, and analysis of data. IJ was involved in

updating, collecting, and early interpretation of data. AC, EC, BJ, KL, ÅL, JN, and TV were involved in study design, data collection, and critically revising the manuscript for important intellectual content. HEL designed the study, was involved in data collection, interpreted data, and contributed to and edited the manuscript. All authors gave final approval of the version to be published.

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Preventing the Predictable

This thesis focuses on several steps in the type 1 diabetes research process. We start by presenting data on early-life risk factors from the DiPiS and TEDDY studies relating to umbilical cord blood autoantibodies, early life stress and the use of analgesic antipyretics. We then present data on the impact of prospective follow-up and study participation regarding status at diabetes diagnosis and during regular clinical follow-up. Finally, results from a clinical trial, DiAPREV-IT, on immune tolerance with GAD-Alum in high-risk children are presented.

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