# Type 1 diabetes in children - risk factors and prediction.

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## - RISK FACTORS AND PREDICTION

Results from the DiPiS study


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# TYPE 1 DIABETES IN CHILDREN

## - RISK FACTORS AND PREDICTION

Results from the DiPiS study

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LUND UNIVERSITY

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Type 1 diabetes in children - risk factors and prediction. Results from the DiPiS study.


Abstract

Susceptibility genes and environmental factors are important for development of Type 1 diabetes (T1D). Increased birth weight (BW), increased linear growth and gestational infections are among the reported risk factors. T1D is predictable by analysis of HLA genotypes and islet cell autoantibodies. In this thesis risk factors and prediction of T1D were studied as part of the Diabetes Prediction in Skåne (DiPiS) study.

In paper I, an analysis of the association between T1D high-risk HLA genotypes, cord blood autoantibodies and BW indicated an increased risk for high relative BW (HrBW) in children with high-risk HLA. Presence of cord blood autoantibodies decreased this risk.

In paper II the effect of gestational infections on BW was studied. Infections with fever and/or gastroenteritis during pregnancy increased the risk for HrBW. Furthermore, infections aggravated, but could not fully explain, the association between high-risk HLA and HrBW. The presence of cord blood autoantibodies decreased the risk for HrBW only when occurring together with infections.

In paper III, intrauterine and childhood growth, corrected for mid-parental height, in children developing T1D were studied in relation to HLA genotypes. HLA genotypes were associated with SDS of birth length, confirming their interaction with intrauterine growth. T1D children were taller, but not heavier, at birth than non-HLA matched controls, but not compared to HLA matched ones. T1D children showed increased height development during the first 18 months compared to both non-HLA and HLA matched controls. Thus, other factors than HLA-genotypes are concluded to be responsible for the increased linear growth before T1D onset.

In paper IV parental reactions to DiPiS was studied. Most parents were not worried by participation in our study.



Key words: Type 1 diabetes, childhood diabetes, HLA, autoantibodies, GAD65, IA-2, IAA, insulin, birth weight, birth length, risk factors, screening, DiPiS

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*To all children in DiPiS and*

*To all children with diabetes*

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# LIST OF PAPERS

This thesis is based on the following original papers, printed by kind permission from Springer Science and Business media and Wiley-Blackwell Publishing Ltd, respectively.

**I** **H. E. Larsson**, K. Lynch, B. Lernmark, A. Nilsson, G. Hansson, P. Almgren, Å. Lernmark, S-A Ivarsson and the DiPiS Study Group.
'Diabetes-associated HLA genotypes affect birthweight in the general population.'
*Diabetologia* (2005) 48: 1484-1491

**II** **H. E. Larsson**, K. Lynch, B. Lernmark, G. Hansson, Å. Lernmark, S-A. Ivarsson and the DiPiS Study Group.
'Relationship between increased relative birthweight and infections during pregnancy in children with high-risk diabetes HLA genotype.'
*Diabetologia* (2007) 50:1161-1169

**III** **H. E. Larsson**, G. Hansson, A. Carlsson, E. Cederwall, B. Jonsson, B. Jönsson, K. Larsson, K. Lynch, J. Neiderud, Å. Lernmark, S-A. Ivarsson for the DiPiS Study Group.
'Increased linear growth in children developing diabetes before 6 years of age in relation to diabetes HLA risk.'
*Submitted*

**IV** B. Lernmark, **H. Elding Larsson**, G. Hansson, B. Lindberg, K. Lynch, S. Sjöblad and the DiPiS Study Group.
'Parent responses to participation in genetic screening for diabetes risk.'
*Pediatric Diabetes* (2004) 5: 174-81

# ABBREVIATIONS

ABIS: All Babies in Southeast Sweden

BDD: Better Diabetes Diagnosis

BL: Birth length

BMI: Body mass index

BW: Birth weight

CI. Confidence interval

CTLA-4: Cytotoxic T-lymphocyte Antigen-4

DBS: Dried blood spots

DiPiS: Diabetes Prediction in Skåne

DIPP: Type 1 Diabetes Prediction and Prevention

DKA: Diabetes ketoacidosis

DPT-1: Diabetes prevention trial - Type 1

EBV: Epstein Barr Virus

FPIR: First-phase insulin response

GAD65Ab: Autoantibodies to glutamic acid decarboxylase

GDM: Gestational diabetes mellitus

GH: Growth hormone

$HbA_{1c}$: Glycosylated hemoglobin

HLA: Human Leucocyte Antigen

HrBW: High relative birth weight

IAA: Insulin atuoantibodies

IA-2Ab: Autoantibodies to insulinoma-associated protein 2

ICA: Islet cell antibodies

IGF-I: Insulin like growth factor

IGF-II: Fibroblast growth factor

INS-VNTR: Insulin gene and the associated variable number tandem repeats region

IvGTT: Intravenous glucose tolerance test

LrBW: Low relative birth weight

MHC: Major histocompatibility complex

MODY: Maturity onset diabetes in the young

MPH: Mid-parental height

OGTT: Oral glucose tolerance test

OR: Odds ratio

PCR: Polymerase chain reaction

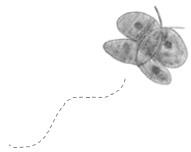
PTPN22: Protein tyrosin phosphatase N22 gene

rBW: relative birth weight

SDS: Standard deviation score

TEDDY: The Environmental Determinants of Diabetes in the Young

ZnT8Ab: Autoantibodies to Zinc T8



# Chapter 1
# BACKGROUND

## Introduction

Type 1 diabetes, or insulin dependent diabetes mellitus, is one of the most common serious chronic diseases in children, with an estimated lifetime risk in Sweden of almost 1 %. The disease is caused by immune-mediated destruction of beta cells in the pancreatic islets resulting in loss of insulin production. Children developing this autoimmune disease become dependent on lifelong treatment with insulin, with enormous consequences for both the child and the family and with a high rate of complications. Years to months before the classical clinical symptoms, including weight loss and increased urination and thirst, an autoimmune sub-clinical destruction of the beta cells has been ongoing. Indeed, at the onset of clinical symptoms, only a small fraction of the beta cells are left. In young children the clinical onset of the disease is often dramatic, and sometimes life threatening.

Despite intense research during the last decades, the aetiology of type 1 diabetes is still unknown. Genetic factors, such as Human Leucocyte Antigen (HLA) genotypes, confer susceptibility to the disease, but other factors are needed to initiate the autoimmune process that results in beta cell death.

In this thesis I will give a summary of the present knowledge of type 1 diabetes aetiology and pathogenesis. Previously unknown mutual relationships between genes, growth and infections during pregnancy as risk factors for the disease will be reported. In addition, I will describe the effects and analyse the possible benefits of screening for type 1 diabetes based on my observations in the Diabetes Prediction in Skåne (DiPiS) study, a prospective population-based study of children with increased risk for type 1 diabetes.

## History of diabetes and the discovery of insulin

The term 'Diabetes', meaning 'go through', refers to the symptom of excess urination (polyuria) and was mentioned already in 164 B.C. in Greece by Galen of Pergamum, who believed that

diabetes was primarily a kidney disease. But long before this, in Egypt 1552 B.C., polyuria was mentioned as a symptom of an unknown disease. 'The melting down of flesh and limbs into the urine', referring to the symptoms of weight loss and polyuria, was a long but quite fitting description mentioned in the 1st century. The term 'Mellitus', meaning honey in Latin and referring to the sweet taste of the urine of the patients, was added in the 11th century, when the patients were diagnosed by the taste of the urine in addition to the classical clinical symptoms. These ancient descriptions of diabetes indicate that the most severe forms of diabetes have affected humans for centuries.

In the 19th century, the first treatment of the disease was introduced. A physician in Paris discovered that the sugar in urine (glucosuria) disappeared during the rationing of food in the Franco-Preussian war 1870-71. This lead to dietary restrictions, which were sometimes very distressing – isolating patients with 'lock and key', very restrictive cures called the 'oat-cure' (almost only oatmeal), the 'milk diet', the 'rice cure', the 'potato therapy', the 'fat diet' and even use of opium. Unfortunately, most patients died despite these treatments.

Paul Langerhans, a German medical student, proclaimed in his thesis of 1869 that the pancreas contained two separate cell systems – one system secreting pancreatic juice and the other with an unknown function. The latter mysterious cell system was subsequently identified as 'islets of Langerhans' and connected with the metabolism of glucose. In 1908, the first injectable animal pancreatic extract to suppress glucosuria was developed, unfortunately with severe side effects. It was not until the summer of 1921 that Frederick Banting and his co-workers Charles Best, James Collip and John Macleod could successfully treat a pancreatectomised dog with an extract of the pancreas containing insulin. In January 1922, a 14-year-old boy in Toronto named Leonard Thompson was the first patient successfully treated with insulin. For this discovery Banting and Macleod were awarded the Nobel Prize in medicine in 1923. Banting shared his prize with Best and Macleod with Collip, the chemist who perfected the extraction method for insulin.

The isolation of insulin is conceivably still the most important discovery in diabetes research, resulting in the successful treatment of a previously lethal disease. Unfortunately, despite the treatment with insulin, it was discovered that diabetes had serious long-term complications affecting the eyes, kidneys, nerves and blood vessels. Studies such as the Diabetes Control and Complication Trial (DCCT) [1] have revealed that glucose control is critical to minimize the risk of diabetes complications. This discovery has resulted in intensified insulin treatment, with a lower rate of complications. New insulin analogues and regimes are tested and continuous subcutaneous infusion using insulin pumps is becoming increasingly widespread. Most recently, continuous glucose monitoring combined with insulin pumps has been introduced to mimic an artificial pancreas. Nevertheless, diabetes is an extremely demanding disease for the patient and society, especially when it comes to children, and indeed also for the entire family.

Although the dietary regimens prior to the discovery of insulin were distressing, they were successful in some cases. These patients were often older and had a 'milder' form of diabetes, leading to the conclusion that there are different types of the disease.

# Types of diabetes

All types of diabetes have one thing in common – high blood glucose (hyperglycaemia). This may be caused by defects in insulin secretion from the pancreatic islet beta cells, defects in insulin action, or both. Presently diabetes is classified into at least four main types: Type 1 diabetes or insulin dependent diabetes mellitus, Type 2 diabetes or non-insulin dependent diabetes mellitus, Gestational diabetes mellitus (GDM) and the Maturity Onset Diabetes in the Young (MODY) [2, 3].

**Type 1 diabetes,** or insulin dependent diabetes mellitus, is characterised by an autoimmune process leading to destruction of the pancreatic islet beta cells. At diagnosis, islet cells antibodies (ICA), or specific autoantibodies to glutamic acid decarboxylase (GAD65Ab), insulinoma-associated protein 2 (IA-2Ab) or insulin (IAA), as markers of the immune reaction, are present in more than 90 % of the patients [4]. Type 1 diabetes accounts for only 5-10 % of all diabetes cases, but is by far the most common form of childhood diabetes. However, type 1 diabetes also appears in young adults, often misclassified as type 2 diabetes due to the commonly slow progress. In this case it is called Latent Autoimmune Diabetes in Adults (LADA) since these patients have GAD65Ab [2]. At the time when clinical symptoms of type 1 diabetes appear, the capacity to produce insulin has almost ceased, and it is necessary to provide exogenous insulin for the patient to survive.

In **type 2 diabetes,** or non-insulin dependent diabetes mellitus, the patients often have both a defective insulin secretion and a decreased effect of insulin (insulin resistance) leading to an increased demand for insulin. Worldwide, type 2 diabetes is the most common type, accounting for 90-95 % of all diabetes cases. As overweight is a risk factor for type 2 diabetes, this disease entity has the most rapid increase, much due to the increasing incidence of obesity. This is also most certainly the underlying reason explaining why type 2 diabetes is also seen in children today, although still representing a minority of the childhood diabetes cases. In type 2 diabetes, resistance to insulin and a relative, but not absolute, deficiency of insulin together cause hyperglycaemia. An autoimmune destruction of the beta cells as seen in type 1 diabetes is not present in type 2 diabetes. Type 2 diabetes may be treated by diet, oral hypoglycaemic medicines and sometimes insulin [2, 3].

**GDM,** is defined as glucose intolerance that is diagnosed during pregnancy [2] and complicates about 1-2 % of pregnancies in Sweden [5]. The prevalence varies between populations but is higher in countries where type 2 diabetes is common [6]. GDM results from pancreatic beta cells failing to compensate for the increased insulin resistance during pregnancy. Sometimes this may be an undiagnosed type 2 diabetes or type 1 diabetes, but mostly it is a time-limited diabetes, which disappears after delivery. GDM may be treated with diet or insulin depending on the severity of disease. Mothers with GDM have a markedly increased risk of developing postpartum diabetes [7].

**MODY** is a heterogeneous group of non-autoimmune diabetes, inherited in an autosomal dominant pattern and accounting for 1-5 % of all cases of diabetes in industrialized countries. MODY is characterized by hyperglycaemia without ketosis or obesity in children or young adults.

The disease is caused by mutations in genes expressed in the beta cells, causing defective beta cell function. Until now, six specific genes have been described: Hepatocyte nuclear factor-4α in MODY 1, Glucokinase in MODY 2, Hepatocyte nuclear factor-1α in MODY 3, Insulin promoter factor-1 in MODY 4, Hepatocyte nuclear factor-1β in MODY 5 and NeuroD1 in MODY 6. In most cases treatment with insulin is not required, but good glycaemic control is necessary to avoid complications [2, 3].

In addition to these four main types of diabetes, there are secondary forms of diabetes that cannot be classified into these groups, namely diseases of exocrine pancreas, drug- or chemical induced diabetes and diabetes associated with excess production of hormones that antagonize insulin action (*e.g.* growth hormone, cortisol, glucagons, epinephrine) [2]. In addition, neonatal diabetes, presenting within the first 4-6 weeks of life, may appear. Neonatal diabetes may be either transient or permanent. It is caused by various birth defects or mutations in genes such as Kir6.2, Glukokinase or Insulin promotor factor-1 or in the 6q24 chromosomal region [3].

The current classification of diabetes into type 1 and type 2 is under contention. In the 'accelerator hypothesis' it is claimed that these two diseases overlap and have a common aetiology based on excess weight gain followed by insulin resistance and successive destruction of the insulin producing cells, as described later [8].

## How is diabetes diagnosed?

Internationally, diabetes is diagnosed according to the following criteria developed by the World Health Organization (WHO) and the American Diabetes Association (ADA) [2], and also recommended by the International Society for Pediatric and Adolescent Diabetes (ISPAD) [9]:

1) A casual plasma glucose of ≥ 11.1 mmol/L and symptoms of diabetes (polyuria, polydipsia, unexplained weight loss)
   OR
2) A fasting glucose of ≥ 7 mmol/L.
   OR
3) A plasma glucose of ≥ 11.1 mmol/L 2 hours after glucose load in an oral glucose tolerance test (OGTT).

In the absence of unequivocal hyperglycaemia, the result must be confirmed on a subsequent day either by repeated sampling as above or by an OGTT.

This definition of diabetes may be questioned in some cases, since an early type 1 diabetes sometimes presents only as a pathological plasma glucose level after a meal, while fasting glucose remains normal and no classical symptoms are present. In children participating in screening studies, where individuals at increased genetic risk for type 1 diabetes are followed, two subsequent unambiguously high plasma glucose values in combination with multiple islet cell

autoantibodies may therefore be sufficient to diagnose the disease, even in the absence of clinical symptoms.

# Type 1 diabetes epidemiology

During the last decades a rapid worldwide increase in the incidence, *i.e.* number of cases/100,000 individuals annually diagnosed, of childhood type 1 diabetes has been reported [10-13]. The incidence varies between countries and regions but the highest rate is generally seen in European and North American populations. The relative increase has been more pronounced in populations with low incidence [10]. In Europe, the incidence is higher in the northern part than in the central and southern parts, with the exceptions of the high incidence region Sardinia [11, 13] and conversely, Iceland with one of the lower incidences in the north [12]. The disease is most common in Finland with more than 50/100,000 children annually diagnosed with type 1 diabetes [14], while Sweden is second with 44 children/100,000 per year. About 7,700 children are currently being treated at Swedish pediatric clinics (National registry of Childhood Diabetes, 2007). The lifelong risk of type 1 diabetes may be estimated to approximately 1 %. Type 1 diabetes is increasing most rapidly in the youngest age groups [11-13, 15, 16] (Table 1). Still, the total incidence is increasing with age, with the highest risk of type 1 diabetes onset in the age group 10-14 years [13]. There is currently no explanation for the increasing incidence of type 1 diabetes in children.

Table 1. Reported annual increase in incidence of type 1 diabetes in different populations, age groups and during different time periods.

| Population | Study Years | Annual increase % (95 % CI) 0-14 years | Annual increase % (95 % CI) 0-4 years | Annual increase % (95 % CI) 5-9 years | Annual increase % (95% CI) 10-14 years | Reference |
|---|---|---|---|---|---|---|
| Global | 1960-96 | 3.0 (2.6-3.3) | | | | [10] |
| Europe | 1989-94 | 3.4 (2.5-4.4) | 6.3 (4.1-8.5) | 3.1 (1.5-4.8) | 2.4 (1.0-3.8) | [11] |
| Europe | 1989-98 | 3.2 (2.7-3.7) | 4.8 (3.8-5.9) | 3.7 (2.9-4.5) | 2.1 (1.4-2.8) | [12] |
| Finland | 1965-96 | | 4.2 (3.6-4.9) | 2.5 (2.1-2.9) | 1.3 (0.9-1.7) | [15] |
| Sweden | 1988-97 | 3.3 | 6.3 | 2.7 | 1.7 | [16] |
| Global | 1990-94 | 2.4 (1.3-3.4) | | | | [13] |
| Global | 1995-99 | 3.4 (2.7-4.3) | | | | [13] |
| Global | 1990-99 | 2.8 (2.4-3.2) | 4.0 (3.1-4.9) | 3.0 (2.4-3.7) | 2.1 (1.5-2.7) | [13] |

Type 1 diabetes generally affects girls and boys equally during childhood, although some studies report a slightly higher rate of increased incidence in boys [15]. However, the incidence peak occurs about 2 years earlier in girls (11 years of age) than in boys (13 years of age) [17], which may reflect the earlier onset of puberty in girls. After 15 years of age the incidence is almost twice as high in men as in women [17].

It is still unknown why the incidence of type 1 diabetes is increasing so rapidly. Swedish data from 1983-1998 suggest that the increment in children 0-15 years reflects a shift towards disease onset at younger age, since no increase in the age group 0-35 years was found [17].

However, preliminary data from Finland point towards a concomitant increase in the total incidence of the disease as well as a decrease in age of onset [14]. It is unlikely that the rapid increase in type 1 diabetes is due to altered genes, since genetic changes in a population are usually slow [10]. Furthermore, immigration from countries with low risk of diabetes has not influenced the rising incidence [18]. Thus, changes in environmental factors, or novel such factors, that together with diabetes susceptibility genes trigger the disease are likely to explain the dramatic increase in incidence rate. However, these environmental factors remain to be defined and there are still unresolved issues regarding the pathogenesis and aetiology of the disease.

# Pathogenesis and aetiology

Type 1 diabetes represents the end-stage of the immune-mediated insulitis that eventually results in destruction of the pancreatic islet beta cells. This autoimmune process is thought to be initiated by one or more exogenous triggers in genetically susceptible individuals.

When a child presents with symptoms of diabetes, *i.e.* thirst, increased urination and weight loss, it is estimated that only about 10-20 % of the insulin-producing beta cells are still functioning [19] and insulin therapy is necessary. The clinical presentation is preceded by an asymptomatic period of varying duration of time. In younger children a rapid destruction of the beta cells may lead to clinical diabetes within months, whereas in older individuals the beta-cell destructive process may take years [20]. During the prodromal period, the appearance of diabetes-associated autoantibodies is the first detectable sign of the process. However, it is still not clear whether these autoantibodies have a pathogenic role or are just the consequence of the T-cell-mediated destruction of the beta cells.

**Death of beta cells**

The insulin-producing beta cells, along with other cells producing glucagon, somatostatin or pancreatic polypeptide, are contained in small clusters of 2,000-4,000 endocrine cells in the pancreas, named islets of Langerhans. Since pancreatic biopsies are difficult to perform and considered unsafe, much of our knowledge about type 1 diabetes pathogenesis is based upon a large number of studies in mice and rats and a limited number of pancreases from patients dying close to the clinical diagnosis of type 1 diabetes. Insulitis in diabetes was reported already in 1940 [21]. The first evidence of autoimmune-mediated beta cell death was reported in 1965, when lymphocytic infiltration in the pancreatic islets was found in deceased patients with type 1 diabetes. Lately, pancreatic biopsies in patients recently diagnosed with type 1 diabetes have been performed in Japan. Insulitis dominated by T-cells and macrophages was found in a majority of the patients; this insulitis being associated with the presence of islet cell autoantibodies [22]. However, in a recent study of adult islet cell autoantibody-positive pancreas donors, it was found that only a small fraction of these non-diabetic adults had insulitis [23]. Moreover, none of the donors positive for less than 3 autoantibodies had insulitis, and no decrease in beta cell mass was found in either of them [23]. This suggests that insulitis may predominate in subjects close to the

onset of hyperglycemia and clinical onset of diabetes. Several hypotheses on diabetes pathogenesis have been proposed based on these and other studies in animals and humans.

One hypothesis suggests that one or more triggering events, such as viruses replicating in the beta cells, initiate the beta cells to undergo apoptosis (programmed cell death). This makes beta cell antigens available to cells of the immune system. The antigens are taken up by dendritic cells, exposing antigen peptides on major histocompatibility complex (MHC) class II molecules (also called HLA heterodimers in humans) on the cell surface. The antigen-MHC complex is recognized by specific T-lymphocytes, in particular $CD4^+$ T helper cells that are able to initiate the autoimmune reaction. Cytotoxic $CD8^+$ T-cells directly attack and kill the beta cells. The cytotoxic T-cells are dependent on $CD4^+$ T-cells, which also activate macrophages as well as B-lymphocytes, the latter to produce autoantibodies against the presented autoantigens [19]. This network of cells cooperates to induce a coordinated immune response involving both cells and antibodies.

Another possibility is that apoptosis of beta cells normally occurs in all individuals, but that the process has pathological consequences only in individuals with an autoreactive T-cell repertoire. These T-cells are mobilized by exposed antigens on the beta cells and initiate the autoimmune reaction, perhaps in combination with genetic and environmental susceptibility factors [24].

However, there are still unresolved mysteries. It is not clear why the autoimmunity is directed to the beta cells and why the other endocrine cells within the islets of Langerhans, such as the alpha cells, are not affected. Furthermore, autoimmunity to the Schwann cells surrounding the beta cells has been reported in mice [25], raising additional questions concerning the process, including the possible pathogenic role of the islet cell autoantibodies.

**Islet Autoantibodies**

When the first autoantibodies to pancreatic islet cells were described in 1974 [26, 27], direct evidence for type 1 diabetes being an autoimmune disease was provided. The antibodies, called islet cell autoantibodies (ICA), were detected by indirect immunofluorescence in patients with type 1 diabetes that also had another autoimmune disease. This made the authors conclude that ICA were uncommon in diabetes except in cases with multiple endocrine deficiencies, since previous studies had failed to detect the antibodies [26, 27]. Later, ICA were found in a large series of children with recently diagnosed type 1 diabetes without other coexisting autoimmune diseases [28]. Furthermore, ICA were detected in one patient a year before the onset of clinical diabetes and in one non-diabetic twin that later developed the disease [26], providing the first evidence of an ongoing autoimmune process before the clinical onset of type 1 diabetes. In 1982 the first autoantigen recognized by ICA, a 64 kDa protein, was demonstrated [29]. This was later identified as an isoform of glutamic acid decarboxylase (GAD65) [29, 30]. The second autoantigen recognized by ICA, an isoform of insulinoma-associated protein 2 (IA-2), was identified in 1996 [31]. For the first time, in 1983, antibodies to insulin (IAA) were reported in diabetic patients before the start of insulin treatment [32], suggesting a role for these antibodies in

the autoimmune process of diabetes. This third autoantigen is not detected by the immunofluorescence test for ICA. Most recently, autoantibodies to a fourth islet autoantigen, Zinc T8 (ZnT8Ab), were reported in patients recently diagnosed with type 1 diabetes [33]. This autoantigen is highly beta cell specific.

**GAD65** is present in neurons and pancreatic islet beta cells and is an enzyme involved in conversion of glutamic acid to γ-aminobutyric acid (GABA) – a major inhibitory transmittor substance in the nervous system. GAD65 is encoded at chromosome 10p11 and is a protein of 585 amino acids. The role of GAD65 within the islet cells is still unknown.

**IA-2** is a member of the protein tyrosine phosphatase family. It is localized to the membrane of the secretory vesicles in endocrine and neuronal cells [34]. IA-2 is expressed both in α- and β-cells in the Langerhans cell islets and this 979 amino acid-long protein is encoded on chromosome 2q35. The function of IA-2 is still unknown since it lacks enzymatic capacity due to an amino acid substitution in the catalytic domain. Autoantibodies to IA-2 are directed to the intracellular domains of the proteins [34].

The third autoantigen, **insulin**, is a short protein of 51 amino acids encoded on chromosome 11q15. IAA are often produced as a consequence of insulin treatment in patients with diabetes, and it is therefore generally not useful to measure IAA after exogenous insulin has been given. However, autoantibodies to insulin are also frequently seen before clinical diagnosis and the start of insulin treatment.

The fourth autoantigen, **ZnT8**, is a protein located in the membrane of the secretory vesicles of the beta cells acting as a transporter of zinc ions. Recently autoantibodies to ZnT8 have been found in 63 % of patients with type 1 diabetes, compared to < 2 % in controls, suggesting that they indeed are diabetes-associated autoantibodies [33]. ZnT8Ab are independent markers of islet cell autoimmunity and can be detected in otherwise autoantibody-negative individuals. Furthermore, ZnT8Ab were found to appear and precede disease onset in prospectively studied first-degree relatives of diabetes patients and high-risk individuals from the general population.

However, some patients are positive for ICA but negative for both GAD65Ab and IA-2Ab suggesting that there are additional, as yet undiscovered, antigens covered by ICA. Some of these patients may have autoantibodies to ZnT8. In addition, several other peptides have been proposed as additional type 1 diabetes-related antigens. Further studies will disclose the importance of these new autoantibodies for the development of type 1 diabetes.

More than 90 % of the patients with type 1 diabetes have previously been reported to have autoantibodies to ICA, GAD65, IA-2 or insulin at diagnosis. The corresponding number for the general population is about 1 % [4]. With the addition of ZnT8Ab to the combined analysis of GAD65Ab, IA-2Ab and IAA, the number of autoantibody-negative individuals at onset of diabetes was recently reported to be reduced from 5.8 % to 1.8 % [33]. The absence of autoantibodies at diagnosis may not necessarily mean that beta cell destruction has been mediated by something other than an autoimmune process. Firstly, it is likely that all diabetes-associated autoantibodies have not been discovered yet. Secondly, in 25 % of type 1 diabetes children who were diagnosed without autoantibodies at clinical onset of diabetes autoantibodies were reported

to have been present in their cord blood [35]. This raises the possibility of an autoimmune process early in life that later has decreased in activity, which may explain why some type 1 diabetes children are autoantibody-negative at diagnosis. However, the risk of developing diabetes conferred by the presence of cord blood autoantibodies at birth remains controversial. Autoantibodies to IA-2 and GAD65 were reported in cord blood of 17 % of children who had developed diabetes before 15 years of age compared to 4 % in control subjects [36], all born of non-diabetic mothers, suggesting a possible pathogenic role. In contrast, no increased frequency of cord blood autoantibodies was reported in patients who were diagnosed at 15-30 years of age, which may indicate less influence of perinatal effects on diabetes risk in older patients with type 1 diabetes [37]. In a prospective study of children with high-risk HLA genotypes for type 1 diabetes, the presence of cord blood autoantibodies was not found to increase the risk of diabetes, but the median age for follow-up when these findings were presented was only 5.3 years [38]. However, when investigating offspring of mothers with type 1 diabetes, autoantibodies in cord blood decreased the risk for the child to develop multiple autoantibodies and diabetes within 8 years, suggesting that foetal exposure to autoantibodies may be protective in children born to type 1 diabetes mothers [39].

A majority of cord blood autoantibodies are transferred over the placenta from the mother and persistent positive autoantibodies from birth are very rare [38]. Children born to mothers with diabetes are frequently positive for cord blood autoantibodies. High levels of autoantibodies in a newborn child are strongly correlated to maternal autoantibodies [38, 40]. However, positive cord blood autoantibodies have occasionally been reported even with an autoantibody-negative mother at delivery [36, 38]. Cord blood autoantibodies may be a prognostic marker for type 1 diabetes, but today it is premature to draw definite conclusions regarding their pathogenic role in type 1 diabetes. Several questions remain to be answered. Firstly, it is not clear whether cord blood autoantibodies always originate from the mother or if the foetus itself sometimes may produce autoantibodies. Furthermore, it is still unclear whether autoantibodies in cord blood affect the child's risk of developing type 1 diabetes later in life, and if this risk differs between offspring of mothers with type 1 diabetes and children in the general population. Finally, it is not known whether cord blood autoantibodies interact with HLA genotypes, gestational infections and other risk factors for type 1 diabetes.

**Hereditary factors**

Monozygotic (genetically identical) twins have a higher concordance rate for developing type 1 diabetes and islet cell autoantibodies than dizygotic twins, supporting the importance of genetic factors for the development of the disease [41]. Still, the concordance rate for identical twins has been found to be as low as 21 to 38 % [41-43], although one study with long-term follow-up reported a rate of 70 % [44]. These findings indicate that environmental factors are also of importance. About 85 % of children who develop type 1 diabetes have no first degree relative with the disease [45], which may in part explain why the families often are completely unprepared for the diagnosis. Nevertheless, the risk of developing type 1 diabetes is higher in first degree

relatives than in the general population. The risk of developing type 1 diabetes in a sibling to a child with diabetes is about 6 % [45], but the cumulative risk in the sibling is dependent on the age at diagnosis of the diabetic child [46, 47]. Siblings to children diagnosed at age 0-4 years have a higher cumulative risk of type 1 diabetes (11.7 %) by the age of 20 than children diagnosed at age 5-9 (3.6 %) and age 10-14 (2.3 %) [47]. Among children with diabetes a diabetic father is twice as common as a diabetic mother [48] and the risk of developing diabetes in a child born to a mother with diabetes is 1.3-4 %, while a father with diabetes confers a 6-9 % risk, with an even higher risk if the father was diagnosed before 17 years of age [45, 49, 50]. The reason why the risk of the offspring of a mother is only half that of the risk conferred by a father is still not known, but it is can be speculated that it might depend on induction of immunological tolerance.

**Genes conferring susceptibility**

The inheritance of type 1 diabetes remained a mystery until the identification of two susceptibility gene regions: the HLA genes on chromosome 6 [51, 52] and the insulin region on chromosome 11p15 [53].

The **HLA genes** are the human equivalent of the MHC, a region of DNA that encodes a group of molecules recognizing antigens. These molecules are referred to as MHC molecules and are called HLA heterodimers in humans. The function of the HLA heterodimers is to present antigenic peptides to T-cells, an important step in the normal immune response against infections. There are two classes of HLA heterodimers. The class I heterodimers are present on all cells while the class II heterodimers are present on B-cells, macrophages, dendritic cells and activated T-cells in the immune system. The T-cell receptor can only recognize foreign or self-antigens if they are presented in complex with the HLA heterodimer, unique for the individual HLA genotype. The HLA genes are highly variable in the population, making it possible to present many different antigens. The HLA class II heterodimers are encoded by three different genes, inherited from both parents; HLA-DP, DQ and DR (Figure 1). Since two variants of each gene are inherited there are six unique alleles encoding HLA class II monomers in each individual. Furthermore, there is variability in the population for the HLA-DP, DQ and DR genes. Consequently, there is a large variability in HLA class II heterodimer composition both within the same individual and, more importantly, between individuals. The HLA-DQ and HLA-DR genes are the most important susceptibility genes [54-56], accounting for about 50 % of the genetic susceptibility to type 1 diabetes [45]. In addition, a recent study reports that the HLA-A and HLA-B genes encoding for HLA class I heterodimers may also contribute to type 1 diabetes risk [57].

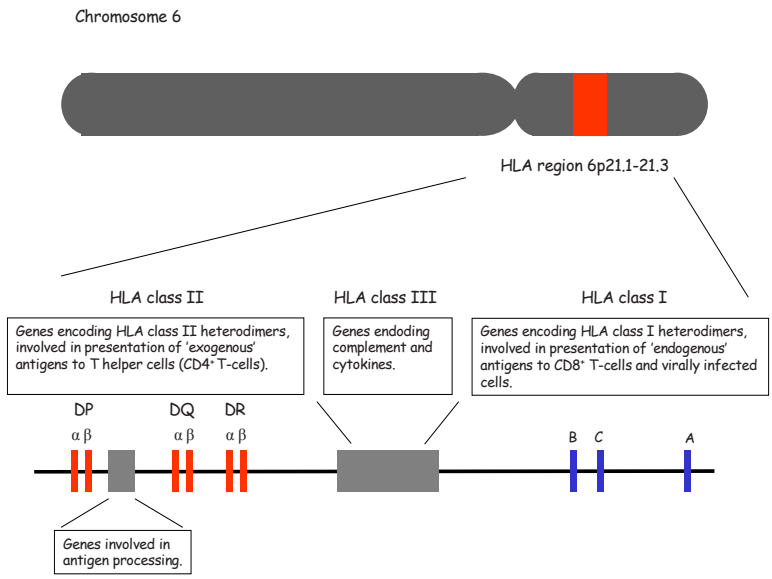



Figure 1. Schematic illustration of the HLA region with the HLA class I, II and III genes encoding their different gene products.

Type 1 diabetes is mainly associated with specific HLA-DQ genotypes [56, 58, 59]. Also specific HLA-DR alleles have been reported to confer risk of developing type 1 diabetes. HLA-DR is tightly linked to specific DQ alleles and this may explain the increased risk. However, HLA-DR has been reported to contribute an additive effect on HLA-DQ through a complex interaction [60] and may not only confer risk of type 1 diabetes by the linkage to high-risk HLA-DQ alleles. The HLA-DQ gene complex is composed of one α- and one β-chain, named by number in order of discovery. There have been multiple studies on the risk of different HLA-DQ alleles throughout the years. Children diagnosed at an early age have a higher frequency of high-risk HLA genotypes than older individuals. Thus, the risk of a specific genotype is age dependent [47, 61, 62]. This may also explain why siblings to children diagnosed with diabetes at an early age have an increased risk, since they are more likely to have high-risk alleles [47]. HLA-DQA1*0301-B1*0302 (DQ8) and HLA-DQA1*0501-B1*0201 (DQ2) are the haplotypes associated with the highest risk for type 1 diabetes. HLA-DQ8 is tightly linked to HLA-DR4 and HLA-DQ2 to HLA-DR3. About 90 % of individuals with type 1 diabetes have at least one of these HLA-DQ alleles, compared with 20 % in the general population [58, 63]. A combination of these two haplotypes confers the highest risk for a child of developing diabetes, constituting a 58 times greater risk compared to the general population [58]. Still, the risk of developing the disease during a lifetime is only 6 % [58] even with this genotype, emphasizing the importance of environmental triggering factors. In addition to HLA-DQ alleles which increase the risk for type 1 diabetes, there are alleles that reduce the risk or even protect against the disease. The HLA-

DQA1*0102-B1*0602 alleles confer a very low risk of type 1 diabetes, even when combined with high-risk DQA1*0301-B1*0302 alleles [58] (Table 2).

Table 2. HLA-DQA1*-B1* genotypes ranked according to type 1 diabetes risk designation. The risk code is an arbitrary numeric description of the risk designation, used in paper III.

| Diabetes risk designation[a] | HLA DQA1*-B1*-genotype | | |
|---|---|---|---|
| | Allele 1 | Allele 2 | Risk code[c] |
| Very high | 0501-0201 | 0301-0302 | 4 |
| High | 0301-0302 | 0102-0604 | 3 |
| High | 0301-0302 | X | 3 |
| Moderate | 0501-0201 | 0102-0604 | 2 |
| Moderate | 0501-0201 | X | 2 |
| Neutral | 0201-0201 | 0102-0604 | 1 |
| Neutral | 0201-0201 | X, 0501-0201 | 1 |
| Neutral | 0201-0201 | 0301-0302 | 1 |
| Neutral | 0102-0604 | X | 1 |
| Neutral | X | X | 1 |
| Neutral | 0301-0301 | 0301-0302 | 1 |
| Neutral | 0301-0301 | 0501-0201, 0201-0201 | 1 |
| Low | 0301-0301 | 0102-0604 | 0 |
| Low | 0301-0301 | X | 0 |
| Very low | 0103-0603 | 0301-0302 | 0 |
| Very low | 0103-0603 | 0501-0201, 0201-0201 | 0 |
| Very low | 0103-0603 | 0301-0301 | 0 |
| Very low | 0102-0602, 0103-0603, 0102-0604[b] | | 0 |
| 'No risk' | 0102-0602 | 0301-0302 | 0 |
| 'No risk' | 0102-0602 | 0501-0201, 0201-0201 | 0 |
| 'No risk' | 0102-0602 | 0301-0301 | 0 |
| 'No risk' | 0102-0602 | X | 0 |

[a] Risk designation criteria used to identify newborns at risk for type 1 diabetes in DiPiS [64].
[b] genotypes DQA1*-B1* 0103-0603/0102-0602, 0103-0603/0102-0604, 0103-0603/X or 0102-0602/0102-0604
[c] Risk code is an arbitrary numeric description of the risk designation.
X is not DQA1*-B1* 0501-0201, 0201-0201, 0301-0301, 0301-0302, 0102-0602, 0103-0603, 0102-0604 but includes potential homozygosity.

HLA genotypes have been used to select risk populations in prospective screening studies for diabetes. Among first- and second-degree relatives, high-risk HLA genotypes are associated with an increased frequency of islet cell autoantibodies [59]. This is also consistent with the finding that the risk of diabetes in a sibling to a diabetic is higher if their HLA genotypes are identical. Earlier studies have reported a risk of 16-30 % for an HLA identical sibling to develop type 1 diabetes [65, 66], but, more recently, a risk as high as 55 % of developing diabetes before the age of 12 has been reported in siblings sharing the high-risk HLA-DQ2/8 with the proband [67]. Furthermore, the risk of persistent autoimmunity in a sibling sharing high-risk HLA with the proband was reported to be 85 % at the age of 15, compared to 20 % for those not sharing both haplotypes [67]. Specific HLA genotypes have also been reported to be associated with different autoantibodies; HLA-DQ2 with GAD65Ab, and HLA-DQ8 with IA-2Ab and IAA [68, 69].

Recently, temporal changes in frequencies of HLA genotypes in patients with type 1 diabetes have been reported in Finland, with lower frequency of high-risk genotypes and higher frequency of low-risk genotypes when comparing individuals developing diabetes in 1990 and 1965 [70]. In the ongoing Swedish Better Diagnosis of Diabetes (BDD) study, where all children diagnosed with type 1 diabetes are genotyped and registered, this drift in HLA genotypes has also been seen. The earlier non-risk genotype HLA-DQA1*0201-B1*0201/A1*0201-B1*0201 now has a significantly increased odds ratio (OR) for diabetes (Table 3) (personal communication). The high-risk genotypes are nevertheless still highly frequent in the youngest age group, while neutral, and even protective genotypes are seen more frequently in older children and adolescents. This may be due to a stronger influence of environmental risk factors triggering disease in older age groups.

Table 3. HLA-DQA1*-B1* genotypes in 994 children developing diabetes in Sweden in the BDD study, compared to 1,011 children typed in the population based DiPiS study (unpublished, 2007).

| HLA-DQA1*-B1*genotype allele 1/ allele 2 | Children with diabetes % (n) | Children in population % (n) | Odds ratio (OR)[a] (95 % CI) |
|---|---|---|---|
| 0501-0201/0301-0302 | 28 % (275) | 3 % (33) | 7.9 (5.1-12.2) |
| 0301-0302/0301-0302 | 11 % (110) | 1 % (15) | 6.9 (3.8-12.5) |
| 0301-0302/0102-0604 | 5 % (53) | 1 % (11) | 4.6 (2.3-9.1) |
| 0301-0302/0101-0501 | 10 % (99) | 3 % (29) | 3.2 (2.0-5.2) |
| 0301-0302/0401-0402 | 4 % (44) | 1 % (15) | 2.8 (1.5-5.2) |
| 0201-0201/0201-0201 | 5 % (52) | 2 % (20) | 2.5 (1.4-4.4) |
| 0201-0201/0102-0604 | 3 % (27) | 1 % (12) | 2.1 (1.0-4.4) |
| 0201-0201/0201-0303 | 1 % (10) | <0 % (5) | 1.9 (0.6-5.7) |
| | | | |
| 0301-0301/X | 10 % (100) | 30% (299) | 0.32 (0.22-0.44) |
| 0601,0602,0603/X | 9 % (90) | 36 % (365) | 0.23 (0.16-0.33) |
| 0301-0301/0601, 0602, 0603 | 1 % (5) | 8 % (85) | 0.02 (0.01-0.06) |
| Remaining genotypes | 13 % (129) | 12% (122) | |

[a] OR for developing type 1 diabetes. Remaining genotypes are used as reference.

Several **non-HLA genetic factors** are known to affect the risk of type 1 diabetes. The insulin gene and the associated variable number tandem repeats region (INS-VNTR) may be associated with determining T-cell tolerance to insulin in the thymus [71] and is estimated to contribute to approximately 10 % of the familial clustering of type 1 diabetes [72]. The cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) gene encodes a receptor expressed by activated T-cells, which limits the proliferation of activated T-cells. Mutations in this gene may play an important role in autoimmunity [45]. Polymorphisms in the protein tyrosine phosphatase N22 (PTPN22) gene, encoding a phosphatase suppressing the activation of T-cells, has been demonstrated to be an important risk factor for type 1 diabetes and to other autoimmune diseases [73, 74]. Together, the INS-VNTR, CTLA-4 and PTPN22 genes confer no more than 15 % of the inheritable diabetes risk [71]. In addition, about 15 possible minor susceptibility genes have also been proposed.

**A pathogenic model**

A model of the pathogenesis and progression to clinical type 1 diabetes has been proposed based on our current knowledge (Figure 2). In an individual with genetic susceptibility for type 1 diabetes, most often by HLA-DQ genotypes, a triggering or initiating event damages the beta cell and starts off the autoimmune process. This event may take place during pregnancy or early childhood. The first sign of the autoimmune process is circulating islet cell autoantibodies. However, in order for the process of destruction of the beta cells to progress, other modifying or accelerating factors are likely needed. Some authors also propose that a driving antigen, as in gluten enteropathy, has to be present for the process to proceed [14]. Before the overt signs of type 1 diabetes, reduced first-phase insulin response (FPIR) may be detected in an intravenous glucose tolerance test (IvGTT). The endpoint of the autoimmune process is the symptoms of clinical diabetes, when only about 10-20 % of the beta cell capacity remains.

During the past decades potential initiating, triggering and accelerating events as well as potential driving antigens have been extensively discussed.

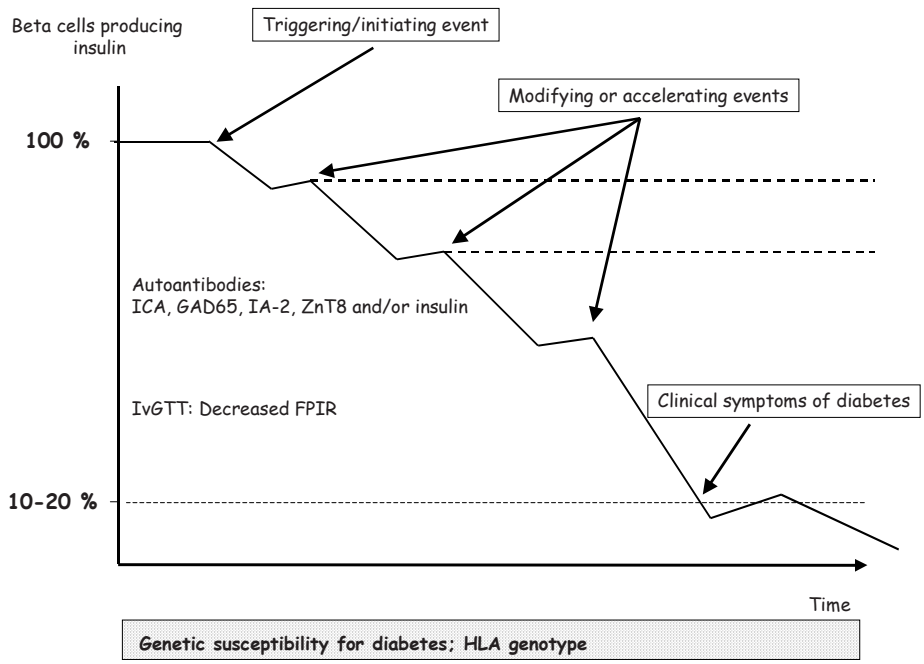



Figure 2. Model of the progression from genetic susceptibility to clinical type 1 diabetes. A triggering or initiating event starts the autoimmune process. Autoantibodies directed towards islet cell antigens are the first sign of the process. Modifying or accelerating events modify the autoimmune process. Decreased FPIR is a late sign, but is measurable before clinical onset of diabetes.
Modified from Knip 2005 [14].

# Environmental factors and triggers

Environmental triggering or initiation factors may occur during pregnancy, at birth, early in infancy or later in childhood. Accelerating or modifying factors are thought to affect the course of the beta cell autoimmunity, and should therefore not appear before the process has been initiated. Consequently, these factors may occur late in pregnancy, in early or late childhood or even in adults, depending on when the triggering event occurred. A potential driving antigen should be present constantly during the autoimmune process, and the process would theoretically stop if the driving antigen were eliminated.

**Pre- and perinatal factors**

Pre- and perinatal factors, *i.e.* factors during pregnancy and shortly after birth, may influence the risk of type 1 diabetes and have therefore been the focus of many studies, sometimes yielding contradictory results.

Low socio-economic status and lower level of education in the mother have been described to increase the risk of type 1 diabetes [75, 76], as well as older age in the mother [77-81] and father [80, 82]. In contrast, a decreased risk of type 1 diabetes has been reported with increasing birth order of the child [77, 80, 82], although the opposite has been reported in one study [79].

The risk of type 1 diabetes has been reported to increase with complications during pregnancy [76, 81], such as preeclampsia [78, 79], caesarean section [77, 78] as well as respiratory distress in the newborn child [79]. The length of gestation has been discussed and found to be associated with risk of type 1 diabetes in some studies. An increased risk with a gestation > 41 weeks was reported in one study [76], while another study suggested an increased risk with low gestational age [83]. Furthermore, in one study an association between the length of gestation and HLA risk genotypes in the child was reported, with a shorter duration of gestation if the child carried high-risk HLA for type 1 diabetes [84].

Maternal blood group incompatibility, leading to hyperbilirubinemia (jaundice) in the newborn child, has also been reported as a risk factor [78, 79]. This was not found when jaundice was present in spite of compatible blood groups [78]. It has not been clear if the hyperbilirubinemia or the treatment of such a condition is responsible for the increased risk, since treatment with phototherapy has been reported to convey an even higher risk [79]. However, increased levels of autoantibodies to ICA and IA-2 have been found in cord blood from children with hyperbilirubinemia following AB0 immunisation, *i.e.* incompatibility between the child's blood-group A or B and the mother's blood-group 0, during pregnancy [85]. This suggests that the condition itself and not only the treatment may be important for the risk.

Many of the pre- and perinatal factors reported to increase type 1 diabetes risk are stress events for both the mother and the foetus, supporting the speculation that stress may be responsible, at least in part, for the increased risk.

**Stress**

Stress is reported to be an increasing problem in our current society, but as early as the 17th century an association between stress and type 1 diabetes was proposed (Willis 1674). Meta-analysis of retrospective case-control studies [86] has indicated that stress and psychological factors may increase the risk of type 1 diabetes [87-90], although this is not supported by all studies [91]. However, it is not trivial to measure stress objectively, especially not in retrospective studies where recall biases are common. Therefore, prospective population-based studies that continuously measure family stress are of importance. In the prospective All Babies In Southeast Sweden (ABIS) study, which prospectively follows 17,000 children born 1997-1999, a mother's experience of divorce and violence was found to be associated with positive IA-2Ab in 2 ½ year old children, suggesting that stressful events can either induce or accelerate autoimmunity [92]. Furthermore, in the DiPiS study, we found a correlation between worries and strong emotional stress during pregnancy in non-diabetic mothers and autoantibodies in cord blood [40]. Stress has several physiological consequences that may be of importance in accelerating or triggering beta cell autoimmunity, such as increased levels of adrenaline and cortisol, which result in increased resistance to insulin. This may lead to increased pressure on the beta cells to produce insulin, leading to initiation of beta cell autoimmunity or acceleration of an already initiated autoimmune process. Stress may also reduce the immune response to infections and thereby, theoretically, increase the risk that viruses may become harmful to the beta cells and trigger an autoimmune reaction.

**Viruses**

The theory that the autoimmune process leading to type 1 diabetes can be triggered and induced by various infectious agents, primarily viruses, has long been discussed. Viruses could either trigger the process *in utero,* by infections during pregnancy, or later in childhood. Different ways of triggering and inducing beta-cell death by viruses have been proposed. Viruses could directly destroy the beta cells by infecting the islet cells in the pancreas, resulting in destruction of neighbouring cells due to cytokine release and exposure of hidden beta-cell autoantigens to the immune system, followed by the initiation of a chronic autoimmune process. Alternatively, viruses could induce the autoimmune process through 'molecular mimicry'. Antibodies produced as an immune response to a viral infection may recognize antigens on the beta cell that resemble viral antigens, leading to a cross reaction and destruction of the beta cells [93, 94] (Figure 3).

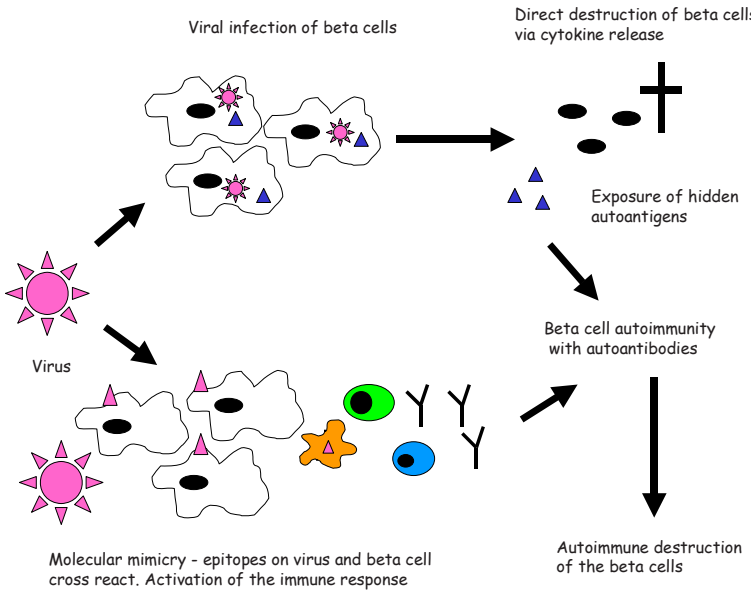



Figure 3. Different ways of triggering and inducing beta cell death by viruses.

One of the first pieces of evidence underlining the importance of gestational infections for type 1 diabetes development was the association between **congenital rubella** and type 1 diabetes described in 1969 [95]. It was reported that almost 20 % of children with congenital rubella developed the disease within the first three decades of life [96, 97]. This provides strong evidence that an infection *in utero* can predispose for the development of diabetes later in life. However, the mechanism is somewhat unclear, as islet cell autoantibodies could not be found in these patients, suggesting non-autoimmune genesis [98]. Congenital infection with **cytomegalovirus (CMV)** has also been suggested to increase type 1 diabetes risk [99], but this has not been confirmed in other studies [100-102]. In a more recent prospective study an association, albeit weak, was found between maternal gastroenteritis during pregnancy and development of GADAb or IA-2Ab [75], suggesting that intrauterine infections could be of importance in triggering the autoimmune process.

Viral infections later in childhood are also suspected to be able to trigger the autoimmune process. Frequent infections before the onset of disease have been reported [103-105]. These could be either pathogenic, initiating the autoimmune process, or, more probably, reflect an accelerated autoimmune process or disclose a subclinical diabetes by the increased insulin requirement that infections usually induce. Moreover, a seasonality in the incidence of type 1 diabetes has been reported, with a lower incidence in the summer [12, 106, 107]. This observaton suggests that environmental factors varying with seasons, such as viruses, may be of importance either in triggering or precipitating the disease. However, this variation is not always obvious in very young children developing diabetes [106, 107]. In prospective studies of children with

susceptibility genes for type 1 diabetes, a seasonal appearance of the first islet cell autoantibodies could be seen. The first autoantibodies more often appear during autumn or wintertime [108] and vary from one year to the next, parallel to the seasonal variation in **enterovirus** infections, supporting the hypothesis that viruses may initiate the autoimmune destruction of the beta cells [14].

Enteroviruses constitute a family that include **Coxsackie** and **echoviruses**, which are common both during pregnancy and infancy. 15 % of expecting mothers have been reported to have been infected with these viruses during pregnancy [109]. Enterovirus infections are frequent in children up to 2 years of age [110], with 43 % of children reported to have been infected before 1 year of age [109]. Usually these viruses cause mild or moderate symptoms from the upper respiratory tract or gastrointestinal tract or they give rise to flu-like disease, but asymptomatic infections are common. More specific symptoms as herpangina, hand-foot-and-mouth disease, myocardial infections and viral meningitis are also seen. *In vitro* studies show that enteroviruses are able to infect pancreatic cells and cause cytolysis [111]. Furthermore, it has been reported that GAD65 shares an antigenic epitope with a protein on the Coxsackie B virus, suggesting that molecular mimicry could be of importance [112].

Children who develop diabetes-associated autoantibodies have been found to have more enterovirus infections as well as higher levels of IgG to echovirus than autoantibody-negative children [110]. A temporal relationship between infections with echoviruses and the appearance of autoantibodies has also been reported [113]. Increased titres of antibodies to Coxsackie B virus have been reported in patients with recently diagnosed type 1 diabetes compared to controls [102, 114, 115], although contradictory results have also been reported [116].

Some strains of Coxsackie and echoviruses can be transmitted across the placenta, thereby infecting the foetus. Significantly increased titers of autoantibodies to Coxsackie or echovirus, suggesting recent infections with those agents, have been found in pregnant mothers of children who later developed diabetes [117, 118]. This indicates a possible pathogenic role in the development of type 1 diabetes, although this finding could not be confirmed by a subsequent study [119].

The role of **rotavirus**, which often causes vomiting and serious diarrhoea, in the pathogenesis of diabetes is unclear. These viruses have proteins resembling epitopes on the major autoantigens IA-2 and GAD65, and could therefore be pathogenic for the beta cells through 'molecular mimicry' (Figure 3) [93]. Although an association between infection with rotavirus and development of islet cell autoantibodies has been reported [120], other studies could not replicate this finding [110, 121].

In a prospective study of children with high risk for type 1 diabetes, infections with either enterovirus, rotavirus or **adenovirus** increased the concentrations of insulin-binding antibodies. Children subsequently developing ICA or IA-2 autoantibodies or diabetes were shown to have a higher concentration of insulin-binding antibodies. Viruses causing diarrhoeal diseases increase the intestinal permeability to antigenic substances, for example bovine insulin from cows' milk. The authors propose that bovine insulin may act as a foreign antigen, triggering the production of

insulin-binding antibodies, thereby preceding the development of beta cell specific autoimmunity [122].

Associations between some other viruses and development of type 1 diabetes have occasionally been described. **Mumps** has been reported to precede the development of ICA and diabetes [123] and **parvovirus B19** was found in one woman developing type 1 diabetes, rheumatoid arthritis and Graves disease [124]. Infection with **Epstein Barr virus (EBV)** has been described to precede the development of diabetes [125] as well as the combination of EBV, Coxsackie virus and adenovirus in a young boy [126].

The role of **retroviruses** in the development of type 1 diabetes is controversial. Retroviruses are enveloped ribonucleic acid (RNA) viruses with a unique capacity for genomic replication. Some species can cause tumours (Human T-lymphotropic virus; HTLV) or Acquired immunodeficiency syndrome (AIDS) (Human immunodeficiency syndrome; HIV). 'Endogenous retroviruses' are integrated in the genome and may encode for superantigens (SAGs), microbial proteins that are able to mediate interactions between the MHC class II molecules and T-cells followed by activation of the T-cells. These 'mobile genetic elements' may either be transmitted as an infection or inherited as a gene and have been suggested to constitute a possible trigger of the autoimmune process leading to type 1 diabetes [127].

The incidence of type 1 diabetes is increasing, parallel to a decreased incidence of infections with enteroviruses [128]. This observation may seem to contradict the theory of viruses as triggers or accelerators of the disease. However, according to the **'hygiene hypothesis'**, initially described in the context of allergies and asthma, a decreased frequency of background infections with microbes in early life due to better hygienic standard could lead to an altered immune response and predisposition to allergic and autoimmune diseases [129]. Indeed, the strongest inverse relation between the prevalence of infectious agents and the presence of atopic sensitisation was seen for enteroviruses [129]. A low exposure to infectious agents may result in decreased immunity among pregnant mothers. This is followed by decreased protection of the child by the mother's transplacentally transferred antibodies, as has been shown for poliovirus. Another example is given by infections with pinworm and helicobacter pylori, which have decreased during the last decades along with the increasing incidence of diabetes [130]. Furthermore, decreased frequency of enteroviral infections might increase the pathogenicity of the remaining viruses. This may be followed by increased sensibility to these viruses in the population and thus a possibility for the viruses to damage the beta cells and trigger the autoimmune disease [128].

**Vaccination**

In contrast to infections, there is no evidence for increased incidence of diabetes following vaccinations [131]. Vaccination to measles, mumps and rubella did not increase the frequency of autoantibodies, in contrast to increased ICA seen in children previously exposed to rubella virus [132]. In one study vaccination to measles was even found to reduce the risk for diabetes [105], though other vaccinations did not affect the risk at all. However, the widespread use of rubella

vaccination has not reduced the increasing incidence rate of type 1 diabetes. Currently, vaccination to rotavirus infections in the general population is under evaluation for cost-effectiveness. Unfortunately, no studies investigating the risk of type 1 diabetes in relation to this vaccination are available so far.

**Dietary factors**

Different dietary factors have been reported to increase the risk for type 1 diabetes. They could theoretically act as potential driving antigens or triggers of the autoimmune process.

In previous retrospective case-control studies an increased risk of type 1 diabetes has been observed if the child had a high intake of **protein** and **nitrosamines** [104, 133], as well as if the mother had a high intake of nitrosamines during pregnancy [76]. These associations have not been confirmed in prospective studies.

Dietary **gluten,** the etiological agent of celiac disease, has been postulated to play a role as a driving antigen in type 1 diabetes. The prevalence of celiac disease in patients with type 1 diabetes was reported to be 4.5 % in the UK [134] but in a Swedish study of 300 diabetes patients the cumulative prevalence was as high as 10 %, compared to about 1 % in the general population [135]. A vast majority of the patients have a 'silent' non-diagnosed celiac disease at onset of diabetes or develop celiac disease within 2 years from the onset of diabetes. It has been suspected that undiagnosed celiac disease could predispose to type 1 diabetes. Removal of gluten in patients with celiac disease was found to lower the frequency of diabetes-related autoantibodies [136] and a gluten-free diet was found to protect against type 1 diabetes development in mice [137]. This could not be supported in the 'Baby-Diab' study, a prospective study of children born to mothers or fathers with type 1 diabetes, where no reduction in autoantibody levels or reduced risk of type 1 diabetes was seen with a gluten free diet in islet antibody positive children [138]. However, supplementation with food containing gluten before 3 months of age in children of parents with type 1 diabetes increased the risk for the child to develop islet autoantibodies, compared to children who were exclusively breastfed until 3 moths of age [139].

**Breastfeeding** has been reported to protect against type 1 diabetes and a short breastfeeding period has been associated with an increased risk of developing the disease [103, 104, 140, 141]. The latter effect could either be due to less immunological protection due to a shorter exposure time to mother's milk or to early introduction of formula based on cows' milk. Nevertheless, the incidence of type 1 diabetes is very high in Sweden and Finland despite the high rate of breastfeeding in these countries.

**Cows' milk** has frequently been proposed either as a trigger of the autoimmune process or as an exogenous driving antigen. Antibodies to a peptide on cows' milk serum albumin have been reported in children with diabetes [142]. In the prospective ABIS-study, introduction of cows' milk during the first 2 months of life was a risk factor for the development of IA-2Ab [75]. In one study, high consumption of cows' milk in childhood was found to be associated with more frequent islet autoantibodies [143] and type 1 diabetes [144] in siblings to diabetes patients. High intake of dairy products before onset of disease has also been reported in some studies [103,

145], but could not be confirmed in another study [133]. The effect of the length of the breast-feeding period and the time of introduction of formulas based on cows' milk on diabetes risk is at present prospectively studied in the international Trial to Reduce Insulin dependent diabetes mellitus in the Generally at Risk (TRIGR). Participating centres in Australia, Canada, Finland, Germany and USA have examined first-degree relatives of patients with type 1 diabetes since May 2002. The children are randomised to receive formula based on either cows' milk or hydrolysed cows' milk upon weaning from breast milk [146]. However, no results from the study are available at present.

The seasonal onset of autoantibodies and type 1 diabetes is not likely to be explained by most dietary components, **vitamin D** being an exception. The sunlight-dependent synthesis of vitamin D in the skin is necessary to achieve sufficient levels of the hormone. The levels are lower in the winter, especially in the northern parts of Europe, where the incidence of type 1 diabetes is high. Apart from the regulatory effects on calcium homeostasis, vitamin D has effects on the regulation and differentiation of the cells in the immune system. The hormone inhibits T-cell proliferation and cytokine production as well as antibody production by the B-cells [147]. Lower concentrations of vitamin D have been reported in blood from children with type 1 diabetes, and lower vitamin D levels during pregnancy have been suggested to increase the risk for the child to develop type 1 diabetes [148, 149]. However, all children in Sweden and Finland are recommended supplementary vitamin D during the first 2 years of life, which should be sufficient to prevent vitamin D deficiency. Nevertheless, supplementary vitamin D has been suggested to protect against type 1 diabetes [150], possibly through the effects on the T-lymphocytes and through suppression of cytokine production [149]. Randomised clinical studies will be necessary to evaluate the effect of increased vitamin D supplementation on the development of autoantibodies and type 1 diabetes in genetically predisposed children.

Supplementation with cod liver oil, an important source of vitamin D and **omega-3 fatty acids**, during the first year of life led to reduced risk of type 1 diabetes in Norwegian children, but no risk reduction was found with other kinds of vitamin D supplementation, suggesting that omega-3 fatty acids were responsible for the effect [151]. In a recent report, the intake of omega-3 fatty acids, adjusted for HLA genotype, family history of type 1 diabetes, caloric intake and intake of omega-6 fatty acids, reduced the risk of developing islet autoimmunity in children [152]. Thus, the anti-inflammatory effect of omega-3 fatty acids may be protective against type 1 diabetes.

One retrospective case-control study of 7-14 year old children revealed that children developing diabetes had a higher energy intake than controls before diagnosis and, independently of this, a higher relative weight. High intake of disaccharides and monosaccharides from milk, bread and sweets was associated with risk for diabetes [153]. However, the increased risk with intake of milk products was not significant after adjustment for energy intake, which suggests that total energy intake and growth were more important for diabetes risk than specific foods.

**Weight and length at birth**

Many studies have focused on birth weight (BW) and birth length (BL) as risk factors for type 1 diabetes. High BW was reported as a risk factor for type 1 diabetes in children developing diabetes up to 15 years of age [82, 83, 154, 155], although this was not supported by a twin study, where neither BW nor BL was associated with type 1 diabetes [156]. In a population-based case-control study, no significant increase in BW in children developing diabetes was found, and a significant increase in BL was only seen among girls [157]. When examining the effect of BW for different age groups at diagnosis, a linear trend of increasing OR for diabetes was found with increasing standard deviation scores (SDS) of BW only in children with onset before 10 years of age [155]. Short BL was found to decrease the risk for type 1 diabetes in children up to 15 years of age [79], while both short BL and low BW for gestational age are known to increase the risk for type 2 diabetes and metabolic syndrome [158-160]. Thus, the 'overfed' foetus seems to have a risk for type 1 diabetes while the 'starved' foetus is at risk for type 2 diabetes.

Foetal growth during pregnancy is influenced both by maternal factors, placental function and foetal factors. Growth velocity is maximal during the second trimester, but maximum weight gain is achieved in the third trimester. Insulin is the most important hormone for promoting foetal growth. Insulin acts through a direct paracrine effect, but insulin growth factor (IGF-I) and fibroblast growth factor (IGF-II) may contribute to the growth [3]. Nutrients from the mother, crossing placenta by diffusion or by active transport, are a rate-limiting factor for growth. Therefore 'starvation' of the foetus will result in a child with low BL and/or low BW if the nutritional deficit occurs early or late in pregnancy respectively. Through metabolic 'programming', the starvation *in utero* is postulated to lead to permanent metabolic changes in the foetus and risk of overweight, type 2 diabetes and metabolic syndrome later in life [161-163]. Conversely, overfeeding the foetus *in utero* will result in a long and heavy newborn child. It is suggested that this may make the beta cells more susceptible to apoptosis and necrosis, thereby increasing the type 1 diabetes risk [164]. However, the evidence for increased BW in children with type 1 diabetes is not as convincing as the evidence for low BW and type 2 diabetes risk. Other factors, *e.g.* genetic factors, could possibly affect intrauterine growth and influence the described association between high BW and type 1 diabetes.

**Childhood growth**

As reported for stress, a high growth rate increases the insulin resistance and the demand for insulin. Therefore, it is suggested that excessive growth may be an accelerating factor in the development of type 1 diabetes. Consistent with this hypothesis is the high peak in incidence of diabetes in puberty, when a high linear growth rate causes an elevated need for insulin due to high caloric intake, while also leading to insulin resistance due to high levels of growth hormone (GH).

During the first year of life infants grow rapidly but at a decelerating rate, both in terms of height and weight. By four years of age the growth rate has declined and remains almost constant, although a seasonal variation may occur as well as a mid-childhood increase in growth,

until the onset of the pubertal growth spurt. Nutritional factors and insulin are the most important growth promoters in infancy. Insulin acts, as in the foetus, through a direct paracrine effect, but IGF-I and IGF-II may also contribute. Between 2-4 years of age, GH in addition to thyroid hormone becomes the major determinant of growth [3].

In several studies, altered growth before the onset of type 1 diabetes has been reported. An increased linear growth up to 7 years before the onset of disease was reported in a retrospective study [165]. In other studies, an increased height and weight SDS from one month of age [166] an increased growth in height between 1-3 years of age [167] and an increment in height and weight before 3 years of age [168] have been shown to increase the risk of type 1 diabetes.

One reason for the increased growth rate in the children who later develop diabetes could be that changes in the metabolism are associated with the autoimmune destruction of the pancreatic beta cells. Others have speculated that type 1 diabetes incidence increases because of altered eating habits in our welfare society [164, 169]. Over-nutrition may cause increased growth in infancy, since obesity in this period of life is likely to result in increased height gain. This could possibly result in increased insulin resistance, mediated via insulin and GH, which in turn would stress the beta cells. In the end this could either trigger the autoimmune reaction via increased apoptosis or accelerate an already initiated autoimmune reaction [164]. Additional stress factors, such as infections or psychological stress, could also be involved in enhancing the insulin resistance. According to the **'overload hypothesis'** up to 20 % of childhood diabetes might be due to alterations in height, weight and body mass index (BMI) [164].

In the **'accelerator hypothesis'** it is suggested that type 1 and type 2 diabetes overlap and have a common aetiology [8]. Three accelerators of beta cell loss are proposed: constitution, insulin resistance and autoimmunity. Neither of these, however, leads to diabetes without excessive weight gain. Thus, weight gain is a central parameter in this hypothesis and it is proposed to cause increased insulin resistance, which would weaken glucose control. According to this hypothesis, insulin resistance, high blood glucose, or both, stress the beta cells and increase beta cell apoptosis, which is further accelerated by autoimmunity in a genetically predisposed individual. Consequently, only the rate of development of diabetes distinguishes type 1 from type 2 diabetes.

Both the 'overload hypothesis' and the 'accelerator hypothesis' are consistent with the reported increase in weight and BMI in children who develop type 1 diabetes [157, 166-168, 170, 171]. An increased BMI was found to be associated with an earlier onset of diabetes [170] and with IA-2Ab at clinical diagnosis [167]. In one study, an increased risk of diabetes by 50-60 % was found in children with 10 % increment in relative weight before 3 years of age, and by 20-40% in children 3-10 years of age [168]. However, the results are controversial and there is no unequivocally convincing evidence for either the overload or the accelerator hypothesis. In some studies, no increase in weight gain or BMI was found in children with diabetes [172-174]. Furthermore, a decrease in BMI at one year of age was seen in the Danish population, despite an increasing incidence of type 1 diabetes [157]. The results are further confounded by the use of data collected from patients after diagnosis of diabetes when insulin treatment had already been introduced [170, 173, 174].

### Genetic factors and growth

Both foetal growth and growth later in life can partly be predicted by the height of the parents, since the mid-parental height (MPH) correlates with both BL and postnatal height development in childhood [175, 176]. Thus genetic factors, such as genes encoding IGF-I, IGF-II and insulin, may affect both intrauterine and childhood growth. A study on the relationship between the INS-VNTR genotypes and size at birth indicated that INS-VNTR III, which confers protection against type 1 diabetes, was associated with both higher BW and BL [177]. In another study of 929 children, the relationship between diabetes associated HLA genotypes and BW was investigated, since such an association could potentially explain the increased BW in children developing diabetes [178]. However, children with the type 1 diabetes high-risk HLA-DQA1*0501-B1*0201/A1*0301-B1*0302 genotype were found to have lower BW than children with the protective HLA-DQA1*0102-B1*0602/A1*0102-B1*0602 genotype, contradicting the hypothesis that the increased BW in diabetic children is a result of a relationship between high-risk HLA and BW. In this study, BW was not corrected for gestational age, but no association between HLA-DQ genotypes and gestational age was found. In contrast, children with the low risk HLA genotype HLA-DR13 (linked to HLA-DQA1*0103-B1*0603) were reported to have an increased relative BW (rBW; BW related to a reference population) [179]. The authors suggest that HLA or some unknown factor linked to the HLA region on chromosome 6 might affect normal growth, or that the association could be due to mechanisms of handling infections during pregnancy since HLA-DR13 protects against certain infectious diseases.

Even if these two studies do not directly support the hypothesis that HLA genotypes conferring risk for type 1 diabetes also confer the increased BW in children developing diabetes, the mere association between HLA genotype and BW suggests that HLA genotypes can affect foetal growth. If this is the case, it is not unlikely that these genes also affect growth in childhood.

## Prediction and prevention of type 1 diabetes

### Prediction

From the late 1970´s and onwards several studies have indicated that it is possible to predict type 1 diabetes among first-degree relatives by analysing HLA genotypes and islet autoantibodies. However, since only about 15 % of children developing type 1 diabetes have a first-degree relative with the disease [45], screening of the general population is necessary in order to find a majority of the children who will develop diabetes. Several such studies, utilizing HLA genotyping and islet autoantibody analysis as predictors for type 1 diabetes, are ongoing (Table 4). By prospective screening studies it may be possible to define the environmental factors that trigger and accelerate the development of type 1 diabetes. The studies enable us to follow the autoimmune process from start until the clinical onset of type 1 diabetes and will help us to understand the course of events. With the results from such studies the knowledge of the autoantibody pattern during subclinical disease and the predictive value of islet cell autoantibodies have been improved.

| Study | Screening period | Screening population | Country |
|---|---|---|---|
| German Baby Diab | 1989-2000 | Offspring to diabetics | Germany |
| Australian Baby Diab | 1993 | First-degree relatives | Australia |
| Diabetes autoimmunity study in the Young (DAISY) | 1993- | First-degree relatives and high-risk children in general population | Colorado |
| Diabetes Prediction and Prevention Program (DIPP) | 1994- | High-risk children in general population | Finland |
| Diabetes Evaluation in Washington (DEW-IT) | 1995- | High-risk children in general population | Washington |
| Prospective Assessment of Newborns for Diabetes Autoimmunity study (PANDA) | 1997- | High-risk children in general population | Florida |
| All Babies in Southeast Sweden (ABIS) | 1997-1999 | General population | Sweden |
| Diabetes Prediction in Skåne (DiPiS) | 2000-2004 | High-risk children in general population | Sweden |
| The Environmental Triggers of Type 1 Diabetes Study (MIDIA) | 2001- | High-risk children in general population | Norway |
| The Environmental Determinants of Diabetes in the Young (TEDDY) | 2004- | Multicentre study of high-risk children in general population | Sweden, Finland, Germany, Colorado, Columbia, Georgia, Washington |

Table 4. Some of the presently ongoing screening studies for type 1 diabetes in first-degree relatives or high-risk children in the general population

The appearance of diabetes-associated autoantibodies is the first measurable sign of the autoimmune process that eventually leads to type 1 diabetes. Autoantibody positivity may appear early in life, and has been reported in children as young as 3 months [108, 180]. The autoantibodies appear sequentially, with the appearance of additional autoantibodies usually taking place within a year after the detection of the first one [14, 181]. While a single autoantibody may be harmless and often represents non-progressive beta cell autoimmunity, the appearance of multiple autoantibodies most often reflects a progressive process [59, 182-185]. The number of detectable autoantibodies is unequivocally related to the risk of type 1 diabetes, both in first-degree relatives and in the general population. In studies of family members of diabetes patients 60-100 % of individuals with three or more autoantibodies develop clinical diabetes over the next 5-6 years, and population-based studies indicate that the risk is similar in the general population [186-188]. The antibody pattern differs between age groups, with IAA being the most frequent antibody in the very young children [62] as well as the first one to appear [108, 180]. IAA sometimes fluctuates and may become negative in sequential testings, but a high titre predicts persistent positivity [183]. On the other hand GAD65Ab is the most frequent antibody in older patients, adolescents and young adults, whereas positivity to IAA and IA-2Ab decreases with increasing age of onset of diabetes [68]. Of these three autoantibodies, IA-2 seems

to be the most specific predictor of progression to type 1 diabetes [69, 180, 183, 189] and is also less often transient [184]. It has been suggested that GAD65Ab rather is a marker of general unspecific autoimmunity, while IA-2Ab and IAA are more specific markers for beta cell death [69]. The recently discovered ZnT8Ab has been reported to generally appear after 3 years of age and thereafter to increase in frequency with age up to adolescence. It can precede disease debut by many years, but its titre is generally sustained during this period of time [33]. Indeed, the titres of all autoantibodies seem to be important, with higher levels predicting risk of persistent autoimmunity and type 1 diabetes [182, 184, 185, 189], especially in combination with a high-risk HLA genotype [184, 190].

Most of our ideas on how to predict type 1 diabetes have been generated from studies of **first-degree relatives** of diabetes patients. In siblings of diabetes patients HLA genotypes, number of islet autoantibodies and the titre of IA-2 are of predictive value [189]. HLA-identical siblings are at greater risk of developing autoantibodies and type 1 diabetes than non-identical siblings [67, 190]. Children progressing to type 1 diabetes have been reported to have more antibodies, higher titres of ICA, IA-2Ab and GAD65Ab and decreased FPIR in response to IvGTT. Therefore, a combination of antibodies and FPIR could potentially predict diabetes [182]. In this study, siblings of diabetic children were examined for factors indicating pre-diabetes at the time when the index case was diagnosed. If pre-diabetes was defined as presence of autoantibodies, 36 % of the children progressed to clinical diabetes, and this number increased to 56 % if FPIR was also taken into account. The prospective observation period had a median of 3.6 years. A young age, a strong humoral response and reduced FPIR seemed to charachterize individuals with a progressive process [182]. In the Diabetes Prevention Trial-Type 1 (DPT-1) the progression of type 1 diabetes was studied in relatives of type 1 diabetes patients. Children developing diabetes had gradually deteriorating glucose tolerance with declining C-peptide levels 2 hours after a glucose load in OGTT over a period of at least 2 years before onset of disease, despite the fact that fasting C-peptide levels remained stable [191]. In another study a risk score based on BMI, age, fasting C-peptide levels and glucose and C-peptide levels 2 hours after OGTT was proposed in ICA-positive first-degree relatives, suggesting that these variables can strongly predict type 1 diabetes [192].

It has been shown that the disease predictive sensitivity of GADAb and IA-2Ab is similar in the **general population** compared to siblings of children with diabetes, but for each separate antibody positivity, a higher cumulative risk was observed among the siblings. However, double antibody positivity confers a similar cumulative risk among siblings and in the general population [188], with additional predictive information provided by the level of the autoantibodies [184]. In one study of school children the positive predictive value of multiple autoantibodies in the general population was 25-75 %, with a sensitivity of 58-100 %, if HLA genotypes were not taken into account [186]. It has also been found that a normal but rising glycosylated hemoglobin fraction ($HbA_{1c}$), a measure that is proportional to the average blood glucose the previous 120 days, predicts clinical onset of diabetes in autoantibody-positive children [193].

**Prevention strategies**

To date, several studies seeking to prevent or delay the clinical onset of type 1 diabetes as well as attempting to spare or even improve the residual beta cell function in patients recently diagnosed with type 1 diabetes have been performed. Several attempts to prevent type 1 diabetes through dietary interventions have already been described herein. These include eliminating cows' milk in infant formula, removal of gluten from the diet or supplementation with vitamin D and omega-3 fatty acids.

Shortly after clinical onset of diabetes and initiation of insulin treatment, the remaining beta cells often increase their capacity to produce insulin, thereby decreasing the need for insulin treatment. This period of low insulin requirement, called 'partial remission' or the 'honey-moon', may last for months or even years and is advantageous for the patient since it simplifies the treatment and improves the metabolic control, leading to less risk of long-term complications [194]. The 'honey-moon' is longer and appears more frequently in older children and adults than in children diagnosed at a younger age [195]. However, it is known that an early diabetes diagnosis with mild or no symptoms is associated with good residual capacity of the beta cells to produce insulin and a longer 'honey-moon' period [196]. Therefore it was suggested that insulin therapy in individuals with sub-clinical diabetes could be advantageous. In the DPT-1 study first- and second-degree relatives with autoantibodies were treated with insulin subcutaneously and orally to examine if this could prevent or delay the clinical onset of type 1 diabetes. Unfortunately, no effect of this treatment was seen [197]. Similarly, within the Type 1 Diabetes Prediction and Prevention (DIPP) study in Finland, a double blind prevention trial was performed with nasal insulin given to children with genetic risk who had developed autoantibodies, showing no protective effect [198] (Simell, personal communication).

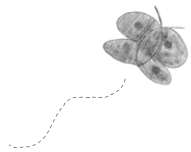
Several ways of affecting the immune system have been tried. Already in the 1980´s plasmapheresis was performed in newly diagnosed type 1 diabetes patients and an improved residual beta cell function and metabolic control was observed [199]. Later, several attempts with immuno-modulating drugs have been carried out. Steroids have been given to suppress the immune reaction, but this treatment had minimal effect, possibly due to their enhancement of insulin resistance. High doses of immunoglobulines also had marginal effects. In the European Nicotinamide Diabetes Intervention Trial (ENDIT), the vitamin nicotinamide was orally administered to autoantibody-positive first-degree relatives of diabetes patients, without any significant effect, despite earlier indications that the drug could protect beta cells [200]. In the Canadian-European Randomised Control Trial Group the immunosuppressive drug Cyclosporin was shown to reduce insulin requirement and enhance the endogenous beta cell function in patients with newly diagnosed type 1 diabetes [201]. Moreover, in a pilot study including autoantibody-positive first-degree relatives of type 1 diabetes patients, with decreased FIPR on IvGTT, a small dose of Cyclosporin was administered. The drug was found to increase the FPIR, suggesting that Cyclosporin may delay the onset of type 1 diabetes in glucose-intolerant siblings [202]. However, the drug has too serious adverse effects, such as kidney toxicity, to be beneficial

[202]. Also immunotherapy to modulate T-cell regulation has been attempted. Anti CD3 antibodies have shown some positive effects in postponing the autoimmune destruction of beta cells [203], but there are serious adverse effects including bone marrow suppression with subsequent serious infections and reactivation of EBV infection with this treatment. Nevertheless, there are some promising ongoing trials with autoantigen therapies. Vaccination with GAD65 has shown promising results with preservation of C-peptide secretion in children recently diagnosed with type 1 diabetes, without side effects [196]. If vaccination with GAD65 can be shown to preserve beta cells it may even prevent disease in children with ongoing beta cell destruction that has not yet progressed far enough to cause clinical diabetes. However, further studies are needed to truly evaluate the effect.

**Psychological aspects of screening**

While parents of first-degree relatives of type 1 diabetes patients may have a clear interest in participating in screening of the disease, it is less apparent why parents of children in the general population would participate in such screening. At the moment the only benefit of screening is the possibility for the child to be diagnosed at an early stage of disease and for the parents to be prepared for the diagnosis. However, an early diabetes diagnosis may be of benefit to the child, especially for the very young children, since children who develop diabetes before 2 years of age are at higher risk of severe metabolic decompensation and diabetes ketoacidosis (DKA) at diagnosis [62]. As many as 40 % of the diabetes children below 2 years of age had DKA at clinical onset of disease in one study [204], further underlining the importance of diagnosing young children early to prevent this life threatening condition. As mentioned, an early diabetes diagnosis with mild or no symptoms is also associated with good residual insulin producing capacity of the beta cells and a longer 'honey-moon' [196]. However, even with this in mind and a probable future possibility to delay or prevent type 1 diabetes onset, screening in the general population at present may still be questioned [205]. Since only about 6 % of children with the highest diabetes risk HLA genotype will develop diabetes, screening children based on high-risk HLA will include many children who will never develop autoantibodies or diabetes. Therefore it is important to monitor the psychological reactions of parents and children in such studies, to ensure that there are no serious consequences for the life of the family. In this context, studies on the psychological reactions to the information about increased diabetes risk in the child have been performed with first-degree relatives of diabetes patients. Information about the presence of islet cell autoantibodies in the child resulted in a transient increased anxiety within the family, which disappeared after 4 months [206]. In contrast, in two population-based studies screening for diabetes high-risk genotypes in newborns, there was no difference in anxiety between parents who were informed that their child was at increased risk of developing diabetes and parents of children without increased risk [207, 208]. However, one of the studies revealed that worrying and depressive feelings do occur in some of the families and that high levels of anxiety were often linked to other stress factors in life [207]. Mothers were more worried than fathers about diabetes risk [207]. Screening for diabetes in the Swedish ABIS-study, where information about

diabetes risk in the child was given to the parents only if requested, revealed an increased anxiety in 1.5 % of the mothers in the general population and 2.5 % of mothers with type 1 diabetes in the family [209]. On the other hand, a majority of the mothers felt calmer when participating in the study [209]. Most studies of parental emotional reactions in screening studies for type 1 diabetes have focussed primarily on the mothers, even if the fathers' reactions are also important for the family situation. In maintaining longitudinal studies it will be important to ensure that the families feel confident and that anxiety is minimized. This might be achieved by providing correct and objective information as well as professional guidance, and by actively involving both parents in the study. Therefore it seems important to investigate both parental reactions to screening for risk of type 1 diabetes.



# Chapter 2

# AIMS

The overall aim of this thesis was to examine and clarify genetic and environmental risk factors for type 1 diabetes, and the psychological and beneficial aspects of screening for this disease.
The specific aims were to:

1) Investigate if the increased risk of type 1 diabetes seen in children with high BW could be an effect of the diabetic HLA risk alleles influencing intrauterine growth.

2) Investigate if infections during pregnancy affect BW and if HLA genotypes and infections during pregnancy interact to influence intrauterine growth.

3) Investigate the joint relationships between infections during pregnancy, cord blood autoantibodies and BW, and their influence on the risk for the child to develop type 1 diabetes.

4) Investigate if HLA genotypes influence intrauterine growth in children developing diabetes before 6 years of age, and if this can explain the previously reported increased risk of diabetes with high BW.

5) Investigate if height and weight development up to 18 months of age are altered in children developing diabetes before 6 years of age, and if these parameters are related to HLA genotypes.

6) Investigate psychological responses of both parents to screening for type 1 diabetes in a prospective population-based study.

7) Elucidate the potential benefits of screening for type 1 diabetes in the general population.

# Chapter 3

# STUDY DESIGN AND POPULATION

## DiPiS

DiPiS is a prospective population-based study of diabetes in children [210]. The aim of the study is to determine the predictive value of genetic risk combined with islet cell autoantibody markers for type 1 diabetes and to identify factors before, during and after pregnancy that may trigger type 1 diabetes in children. In DiPiS, newborn children in the general population are screened for type 1 diabetes high-risk genes. Children with increased risk of type 1 diabetes primarily based on the HLA genotype of the child, are thereafter followed from 2 to 15 years of age. The study has been approved by The Ethics Committee at Lund University, Sweden.

All parents of children born between September 2000 and August 2004 in Skåne, the most southern part of Sweden, were invited to participate in the study. About 12,000 children are born annually in Skåne at the five maternity clinics in Malmö, Lund, Helsingborg, Kristianstad and Ystad. The parents were informed about DiPiS during pregnancy at the Maternity Health Care Clinics (Mödravårdscentralerna) orally and by means of video, posters and pamphlets. The parents were also encouraged to visit the homepage (http://www.med.lu.se/DiPiS) of the project for more information about diabetes and DiPiS. After oral consent, at delivery, a sample of umbilical cord blood was drawn from the newborn child as well as a venous sample from the mother.

When the child was around 2 months of age the parents received a letter of invitation to participate in DiPiS. The participating parents gave their written informed consent and filled out two questionnaires. In the 'Hereditary questionnaire' the parents reported heredity for diabetes by indicating if any family members had been afflicted by the disease. This form also asked for data about birth weight and length of the child. The 'Psychosocial questionnaire' included questions about pregnancy, delivery and the 2 first months of the child's life. In this questionnaire the mothers answered two questions on infections during pregnancy, including infectious diarrhoea, vomiting and febrile illness (>38° C), frequency of illness, and during which trimester the symptoms appeared. In addition, the questionnaire also contained five identical questions to be

answered by both mother and father. These questions were included to obtain information about both parents' views on issues that could be relevant for participation in the study, and contained information about each parent's level of satisfaction with the information about the study, the knowledge of type 1 diabetes before enrolment as well as their concerns about the child's risk of developing diabetes in the future.

A total of 35,683 cord blood samples, from 48,058 newborn children (74 %), were drawn at the maternity clinics in Skåne during the 4 screening years of DiPiS. Cord blood was analysed for HLA genotypes as well as presence of GAD65Ab, IA-2Ab and IAA (se Chapter 4; Methods).

Participating parents of children who had increased risk of developing type 1 diabetes were contacted again for a follow-up when the child had reached 2 years of age. This risk was primarily based upon the HLA genotype of the child, but other risk factors for diabetes were also taken into account. These included heredity, islet cell autoantibodies in cord blood, BW for gestational age (children born large for gestational age; LGA) and infections during pregnancy. Based on these criteria, approximately 6,000 of the 35,683 children were selected for invitation to participate in the follow-up in DiPiS, of which 3,680 children are presently followed in the study. The remaining families either chose to abstain from participation or dropped out after one or more of the yearly follow-up contacts.

When the child was 2 years of age, the parents were also asked if they wanted to be informed about the child's risk of developing type 1 diabetes. If they agreed, written information was sent out by regular mail. We did not have the possibility to inform the parents of the child's risk of developing type 1 diabetes until 2004 because of a decision by The Ethics Committee. Therefore some parents did not receive the information about this risk until their child was 3 years of age. However, if parents had requested the information about type 1 diabetes risk before that, they were given the result.

For the 3,680 children participating in the DiPiS follow-up, annual blood samples are analysed for islet cell autoantibodies and the parents fill out new questionnaires yearly. These contain, among other things, questions about nutrition, diseases, medications, stressing events in the family and a follow-up of the parental reactions to the study. If the children develop autoantibodies, the parents will be contacted if they have agreed to be informed about risk. Children who develop more than one autoantibody are followed every third month at their local hospital by paediatricians linked to the study. At each visit the child's length and weight and information about diseases, medications and life events during the past 3 months are recorded. A blood sample is taken for analysis of islet autoantibodies, $HbA_{1c}$ and random plasma glucose. Children with multiple islet autoantibodies are also offered OGTT every six months. We are planning to follow the children until they are 15 years of age.

All studies in this thesis are based upon participating children in the DiPiS study, with the exception of paper III, in which non-participating children who had developed diabetes and were born within the screening years (September 2000-August 2004) of DiPiS were additionally included.

# Paper I

In this study, 16,709 children participating in DiPiS born between September 2000 and December 2003, *i.e.* the initial 3 $^{3}/_{12}$ of the 4 inclusion years of DiPiS, were analysed. During this period, approximately 38,550 children were born in the province of Skåne and a total of 29,547 cord blood samples were obtained at the five maternity clinics. After excluding twins, triplets and children born to mothers with diabetes (2,865 individuals), 26,682 children remained. Of these, 17,527 of the parents completed the questionnaires and consented to the study when their child was 2 months old. Reported BW from the questionnaire and gestational age recorded at birth were available for 16,709 children (62.6 % of the 26,682 eligible children).

Since all parents did not fill out the questionnaires we compared participating with non-participating children by using maternal information recorded at birth. Participation was higher with increasing age of the mother and with increasing gestational age. There was no difference in frequency of diabetes high-risk HLA or positive autoantibodies between the groups. Among the 16,709 newborn children, 51.3 % were boys and 48.0 % were girls (information of gender was missing in 0.7 % of cases).

# Paper II

Children born within the whole inclusion period of DiPiS, September 2000 to August 2004, were included in the analyses for this study. Of the 35,683 cord blood samples obtained, 31,446 children remained after exclusion of twins, triplets, preterm children (born before 37 weeks of gestation) and children born to mothers with diabetes. BW, gestational age and HLA were available for 19,756 children (63 %). A limited number of mothers had more than one delivery within the inclusion period of the study. The study design precluded identification of these siblings. As in paper I, participation was higher with increasing age of the mother and with increasing gestational age of the child. In this study the participation was also slightly higher for children with diabetes high-risk HLA, but there was no difference in participation rate depending on if the child had islet autoantibodies in the cord blood or not.

# Paper III

In this study, data from children developing diabetes was analysed. A total of 60 out of 48,000 children (0.1 %) born in Skåne during the inclusion years of DiPiS, September 2000 to August 2004, had developed diabetes as of December 2006.

**Type 1 diabetes children:** The 60 children (35 boys and 25 girls) who developed diabetes before December 2006 were between 9 months and 5 years and 10 months (median: 2.75, range: 0.75-5.84 years) at diabetes diagnosis. As many as 7 boys and 4 girls, *i.e.* 11 children (18 %),

developed diabetes before 18 months of age. A cord blood sample for the DiPiS study had been obtained at delivery from 50 of the children. 14 children with high-risk HLA genotypes and participating in DiPiS developed diabetes before 2 years of age. Since the follow-up in DiPiS was offered from 2 years of age, these 14 children had, unfortunately, no opportunity to be followed (Figure 4). Gestational weeks ranged from 30 to 42 weeks and 3/60 children were born prematurely (one child born at 30 weeks of gestation and 2 children born at 36 weeks of gestation). An additional three children were born to mothers with gestational diabetes but none of the children had a mother with type 1 diabetes. After written consent, growth curves were obtained from 58/60 children. 34 of the 60 children who developed diabetes participated in DiPiS (Figure 4).

**Control children:** A total of 155 controls, all born to non-diabetic mothers, but matched for gender, gestational week and date of birth (year and month and as near date as possible) were randomly selected from the 25,378 children participating in DiPiS. Among the control children, 51 were HLA matched to the diabetes children. Growth curves were obtained after written consent.

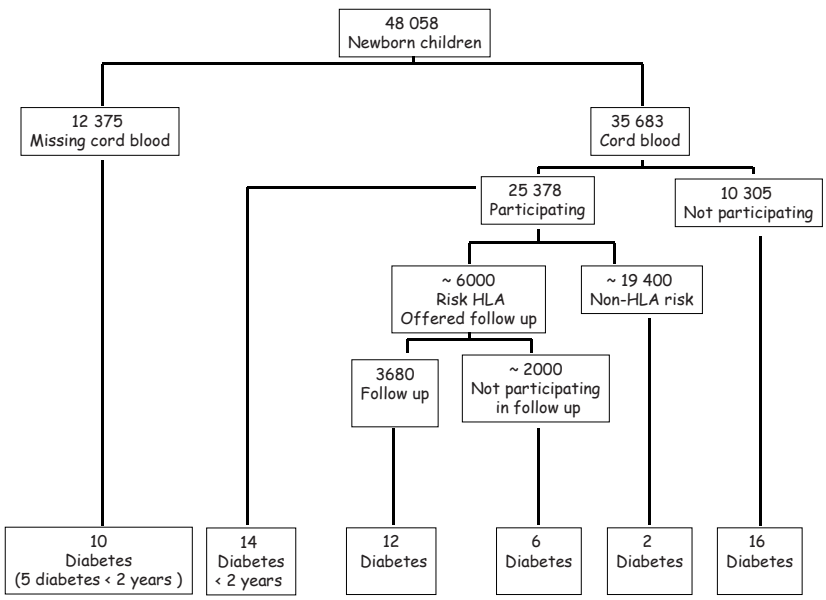
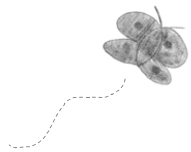



Figure 4. Children developing diabetes participating and not participating in the DiPiS study. Since children with high risk for type 1 diabetes were offered follow-up from 2 years of age, 14 children participating in DiPiS and developing diabetes below this age did not have the opportunity to participate in the follow-up.

# Paper IV

Paper IV is a report that covers the first year of screening in DiPiS, and includes children born between September 2000 and December 2001. This period also included the four 'start-up months', when sampling at birth was not performed at full rate at all the maternity clinics. During this period cord blood samples were obtained from 10,856 children, of a total of approximately 14,100 deliveries, including 154 pairs of twins and two pairs of triplets. A total of 6,831 families (64.8 %) gave written consent to participation in the study. For 6,676 of the participating children the psychosocial questionnaire was filled out.



# Chapter 4
# METHODS

All laboratory analyses in this thesis were performed within DiPiS at the same laboratory and with the same methods throughout the study.

## HLA genotyping

HLA genotypes were analysed by polymerase chain reaction (PCR) from dried blood spots (DBS) on filters, as was initially described by Sjöroos *et al.* and Ilonen *et al.* and later modified by Nejentsev *et al.* [211-213]. Filter samples of DBS were punched into PCR plates. HLA-DQB1 alleles were amplified with sequence specific primers. The product was transferred to streptavidin-coated microtitre plates and diluted with DELFIA hybridisation buffer (PerkinElmer Life Sciences, Boston, MA, USA). After incubation and denaturation, the single-stranded DNA was hybridised with two sets of probes; the first set containing Eu-DQB1*0602/3, Sm-DQB1*0603/4 and Tb-Control and the second one containing Eu-DQB1*0302, Sm-DQB1*0301 and Tb-DQB1*02. After washes and addition of DELFIA enhancement solution (PerkinElmer Life Sciences, Boston, MA, USA), the Eu and Sm fluorescence was measured in a Victor2 MultiLabel Counter (Perkin Elmer Life Sciences, Boston, MA, USA). The Tb signal-to-noise ratio was calculated with MultiCalc, a computer program provided by the manufacturer. The samples positive for DQB1*02 were further analysed for DQA1*0201 and 05 alleles to separate subjects with DR3 from those with DR7. HLA-DQA1 typing was performed with the same technique as for DQB1 typing, with some modifications, using streptavidin-coated plates, dilution with DELFIA hybridisation buffer and the use of Sm-DQA1*05 and Tb-DQA1*0201 probes.

For children in paper III, who had developed diabetes but from whom no cord blood had been drawn at delivery, HLA as well as autoantibodies were determined in venous blood taken at time of diagnosis. The HLA genotype was determined using the same procedure as described above for children participating in DiPiS.

In papers I and II the HLA genotypes were divided into seven risk groups for type 1 diabetes: Very high risk, High risk, Moderate risk, Neutral risk, Low risk, Very low risk and 'No risk', respectively (Table 2). An arbitrary risk code based on the type 1 diabetes risk of different HLA-genotypes was constructed for paper III (Table 2).

## Autoantibodies to GAD65 and IA-2

Autoantibodies to GAD65 and IA-2 were analysed by means of a radioligand binding assay, as described by Grubin *et al.*, Verge *et al.* and Hampe *et al.* [214-216]. First, a combined screening was performed as previously described [215]. Eluates from DBS were incubated with labelled antigens of both GAD65 and IA-2. Antibody-bound labelled antigen was thereafter separated from free antigen by Sepharose-Protein A and the radioactivity counted in a Beta Plate Reader (Perkin Elmer Life Sciences, Boston, MA, USA). The combined screen, referred to as COMB, compared a positive reference with two negative samples to determine a cut-off level that would require the sample to be analysed for both GAD65Ab and IA-2Ab in separate assays. Levels of COMB > 95$^{th}$ percentile were considered positive, and re-assayed individually for GAD65Ab and IA-2Ab by separate radioligand binding assays [214, 216]. GAD65Ab > 35 U/ml and IA-2Ab > 6 U/ml were considered positive.

## Autoantibodies to insulin

IAA was analysed by a radioligand-binding microassay adapted to a microtitre plate format requiring only small amounts of blood and developed for the purpose of screening of multiple samples by Williams *et al.* [217]. The assay was not adapted to be performed on DBS, thus serum from the study subjects had to be used. At first a non-competitive screening for IAA was performed. 7 µl of serum was incubated in duplicate wells with 36 µl of labelled insulin for 48 hours. A total of 25 µl of the incubate was thereafter transferred to a coated microtitre plate with Sepharose-Protein A to separate antibody-bound labelled antigen from free antigen and incubated for 1.5 hours. After washing in Tris buffer and addition of scintillation fluid, the plates were counted by use of a 1450 Microbeta counter (Wallac, Åbo, Finland) and the results were expressed as percent of positive controls.

Samples positive for IAA were re-analysed by a competitive radioligand-binding assay. A total of 7 µl of each serum was added to 4 wells on a microtitre plate. Unlabelled human insulin (Actrapid®, Novo Nordisk) was added to two of the wells of each sample. Labelled insulin was thereafter added to all wells. The plates were incubated with Sepharose-Protein A to separate the antibody-bound labelled antigen from the free one. After washing in Tris buffer and addition of scintillation fluid, the plates were counted by use of a 1450 Microbeta counter as above. Specifically bound antigen for each subject sample was calculated by subtracting the radioactivity of the tubes with excess unlabelled insulin from the activity of those with the labelled insulin alone. These values were thereafter related to positive controls.

# Analysis of growth

In papers I and II the BW of the DiPiS children was analysed. The weight and gestational age of the child were obtained from the questionnaires filled out by the parents when the child was 2 months of age.

In paper III the BW, BL, linear growth and BMI of children developing diabetes were compared to matched controls. This was done by use of growth charts from the Child Health Services (Barnavårdscentraler). Swedish children are regularly examined at a Child Health Services until they start school. At each visit, weight and height of the child are recorded, and a growth chart is filled out. Consent was obtained from the parents to obtain these growth charts, which contain BW, BL, parental height and growth in infancy compared to standard Swedish normograms [218]. The growth of the child was also corrected for the MPH, which correlates with both BL and postnatal growth of the child [175, 176], by using information about the parents' heights, which was available from 95 % of the children.

# Statistical methods

All questionnaires and forms received from the subjects participating in the DiPiS study were scanned into a database, BC/OS system, Biocomputing OS 2000 (Biocomputing Platforms Ltd Oy, Espoo, Finland).

In papers I and II, rBW (=BW SDS) was calculated as Z scores for each gestational week and gender by the following formula:

Z score = (BW – population mean [BW])/population SD [BW].

Population means (SD) of BW were estimated internally using the study group. In paper I, the standardized mean BW for each gestational age and gender was also calculated for all newborns using two polynomial equation formulas, described earlier and used as a basis for the standard weights in Swedish maternity clinics [219]. Based on the rBW the children were divided into quartiles, where children in the lower quartile were defined as having a low rBW (LrBW) and children in the upper quartile were defined as having a high rBW (HrBW). Differences in proportions and the linear association of HrBW with number of infections within HLA risk groups were tested using **$\chi^2$-tests**. Associations between cord blood autoantibodies, infections and HrBW were tested using **Simple logistic regression** models. **Multiple logistic regression** analysis was used to test whether diabetes risk HLA genotypes were associated with HrBW or LrBW after adjusting for gender of the child, maternal age, maternal smoking and whether HLA and cord blood autoantibodies were independently associated with HrBW.

In paper III, BW and BL SDS were calculated as described [220]. Height and weight SDS after birth were calculated using data from the Swedish reference population [218]. MPH was calculated according to the following, earlier described, formula [221]:

SDS MPH = (height SDS of father + height SDS of mother)/1.61.

Diff MPH was defined as SDS birth length or SDS childhood height minus SDS MPH.
BMI SDS was calculated according to Karlberg *et al.* [222].

Non-parametric tests were performed. **Mann-Whitney two group's comparisons** and **Wilcoxon signed rank tests** were used to test for changes within groups. In bi-variate correlation analyses, **Spearman´s rho** was applied**.** By Multiple regression analysis we tested if gestational age, diabetes, MPH or HLA high-risk genotypes were associated BL SDS or BW SDS. HLA risk code genotypes 3 and 4 were compared to genotypes 0 and 1 (Table 1) with BL SDS and BW SDS as dependent variables.

In paper IV, $\chi^2$-tests were used to test for associations between two categorical variables. Whether different factors at birth were predictors for participation in the study was examined by logistic regression. **Two sample t-test** was used to test for differences between independent groups and **paired t-tests** were used to compare between mothers' and fathers' answers to the questionnaires. The relationship between parents' worries and potential predictors was analysed using Multiple linear regression analysis.

In all papers p-values $< 0.05$ were considered significant.

# Chapter 5

# RESULTS AND DISCUSSION

## HLA genotypes and BW

The aim of paper I was to analyse if HLA genotypes affected the risk of high BW and if diabetes high-risk genotypes were responsible for the previously shown relationship between type 1 diabetes and high BW. In a study of 16,709 newborn children we observed that children with the diabetes high-risk genotypes HLA-DQA1*0501-B1*0201/DQA1*0301-DQB1*0302 (DQ2/8), DQ8/ DQA1*0102-B1*0604 and DQ8/X had a higher frequency of HrBW.

When using internal Z-scores in our large cohort of term (born after 37 weeks of gestation) children for determining the rBW, and comparing them to Swedish standard foetal weights for gestational age (based upon 759 ultrasonically estimated foetal weights in 1996 [219]), we found that children in our cohort were slightly heavier. This is consistent with the national trend of increasing BW reported by the Swedish Medical Birth Registry, National Board of Health and Welfare [223].

We discovered that HLA genotypes could be ranked according to highest and lowest frequency of LrBW and HrBW, indicating that HLA genotypes interact with foetal growth to affect BW. The HLA types associated with the highest frequency of children with HrBW were also negatively associated with LrBW and the converse. Children with HLA-DQ8 in combination with either HLA-DQ2, HLA-DQA1*0103-B1*0603, HLA-DQA1*0102-B1*0604 or diabetes neutral DQX had the highest frequency of HrBW. All children with DQA1*0103-B1*0603 were more likely to have a low frequency of LrBW.

When analysing HLA genotypes conferring risk for type 1 diabetes and BW, a strong association between the type 1 diabetes risk genotypes and quartiles of rBW ($p=0.01$) was found. Children with the diabetes high-risk HLA genotypes HLA-DQ2/DQ8, DQ8/DQA1*0102-B1*0604 and DQ8/X had an increased frequency of HrBW (OR 1.20, $p=0.0006$) compared to the rest of the population. Also when comparing with diabetes neutral HLA genotypes, there was an increased risk of HrBW in children with HLADQ2/DQ8 (OR 1.32, $p=0.004$). Furthermore, children with the low risk HLA-DQA1*0103-B1*0603 had increased frequency of HrBW (OR

1.13, p=0.025) and decreased frequency of LrBW compared to neutrals (OR 0.88, p=0.03). These observations confirm a previous finding that children with DR13, strongly linked to the DQA1*0103-B1*0603 allele [224] and known to be protective against type 1 diabetes, also had an increased median BW [179]. Regression analysis showed that the HLA-associated increase in BW was independent of confounding factors (gender, maternal and gestational age and smoking).

The reported effects on BW by HLA genotypes were small and therefore not likely to be seen in study populations of a relatively limited size. In a previous study it was reported that the HLA-DQ2/DQ8 genotype had a lower BW compared to DQB1*0602/DQB1*0602, but this conclusion was based on only 969 children [178], which may explain the discrepancy between these investigations.

The correlation between HLA-DQ subtypes and BW might explain the previously reported increase in BW in children who later develop type 1 diabetes. The effect of HLA genotypes on intrauterine growth could be due to an altered metabolic control in mothers with diabetes susceptible genotypes. For example, these genotypes may predispose to subclinical hyperglycaemia during pregnancy and thus an increased BW. Due to this potential strong influence of maternal hyperglycemia and insulin levels on foetal growth, diabetic mothers were excluded from the analysis. It may be reasonable to speculate that the mechanisms coupling HLA genotypes to altered metabolism include different ways to respond to gestational infections, possibly by HLA-dependent differences in the strength of the immune response to the infection. Therefore, in paper II, we wanted to examine how infections during pregnancy were associated with HLA genotypes and BW.

## Infections during pregnancy, HLA and BW

In paper II we further analysed the associations between HrBW and high-risk diabetes HLA genotypes. This was done by testing the hypothesis that the rBW may be affected by infections during pregnancy. We were able to report that there was a significant, but complex, interaction between rBW, infections and HLA.

Of the 19,756 mothers answering the questionnaire, 14.4 % reported fever, gastroenteritis (diarrhoea and/or vomiting) or both during pregnancy. Fever was more frequently reported than gastroenteritis, while 3.0 % of the mothers reported both in the same trimester and 1.7 % reported infections in more than one trimester. The mothers tended to report more infections if they were older (>30 years), better educated, had a previous child or reported a serious or lengthy disease during the past 5 years. The frequency of gastroenteritis reported in our study was only 6.7 % compared with 30 % in another population-based study [225]. This discrepancy may be due to imprecise reporting of infections with either under-reporting of infections in our study or over-reporting in the other study. Another possible explanation could be that our questions to the mothers were distinctly focussed on infectious gastroenteritis and fever, and did not include chronic diarrhoea or nausea during early pregnancy.

Infections during pregnancy were associated with increased risk of HrBW (p=0.0003). No difference was found in OR between the effect of fever (OR=1.17 of HrBW, p=0.002) or gastroenteritis (OR=1.13 of HrBW, p=0.07), but the frequency of infections was of importance. Furthermore, infections during the first trimester (OR=1.22 for HrBW, p=0.005) and second trimester (OR=1.21 for HrBW, p=0.006) seemed to be of greater importance than infections during the third trimester (OR=1.11, p=0.14).

We also analysed if there was a relationship between infections during pregnancy, HLA genotypes and rBW. We found a linear trend with increasing HrBW correlating with more reported infections in children carrying the diabetes high-risk HLA-DQ2/8 and being negative for cord blood autoantibodies (p=0.007). This was true even when the results were adjusted for confounding factors by multiple regression analysis. Furthermore, the association between HLA-DQ2/8 and HrBW described in paper I was markedly enhanced when the mothers reported infections during pregnancy. Children of mothers who had reported infections in more than one trimester had an OR of 5.24 (p=0.003) for HrBW among the HLA-DQ2/8 carriers compared to all other genotypes. Nevertheless, infections during pregnancy could not explain all of the described association between diabetes high-risk HLA genotypes and HrBW, since there was still a weak but significant association even without reported infections (OR=1.22, p=0.04). The non-diabetes risk allele HLA-DQB1*0603 was also, as previously described, found to confer a higher risk of HrBW, but only for children of mothers who had reported several infections during pregnancy (Figure 5).

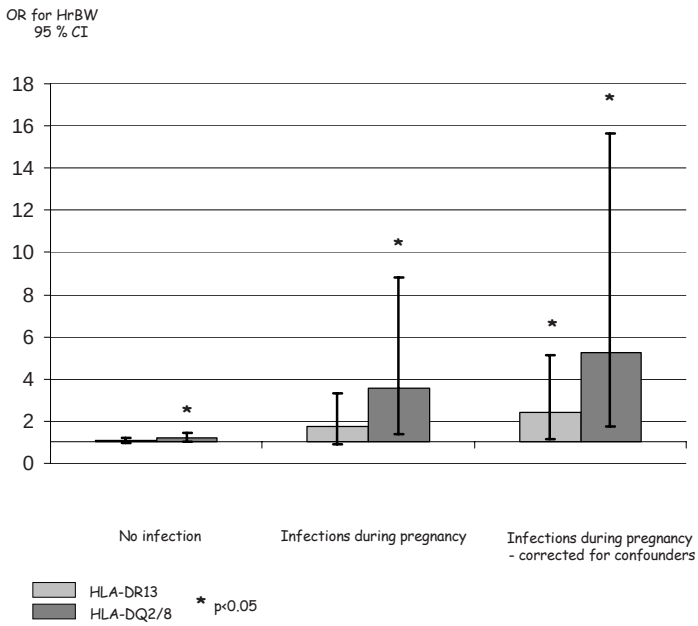



Figure 5. The OR for HrBW in children with HLA-DR13 and HLA-DQ2/8 without and with reported infections during pregnancy, before and after correcting for confounders.

Taken together, our findings that infections during pregnancy increase the risk of HrBW in the child and enhance the association between HLA genotypes and HrBW, suggest that infections may interact with HLA in modifying intrauterine growth. This conclusion is also supported by the observation that also the diabetes low-risk genotype HLA-DQA1*0103-B1*0603 was associated with HrBW (p=0.02), but only in cases with several reported infections during pregnancy. Thus, it cannot be excluded that infections during pregnancy may explain the previously reported increase in BW with the HLA-DQA1*0103-B1*0603 linked DR13 [179]. The remarkable increase in the risk of HrBW in HLA-DQ2/8 positive children born to mothers reporting infections is of considerable interest, as around 30 % of children developing type 1 diabetes have this HLA genotype [68, 226].

At present, there is no simple explanation for how infections and HLA interact with intrauterine growth of the child to affect BW. We propose that antigen-presenting cells in the mother take up certain infectious agents. They are thereafter processed and presented in complex with the HLA heterodimers, thereby inducing an immune response. In this immune response, cytokines are produced, resulting in fever and metabolic changes. It is reasonable to speculate that the well-known insulin resistance during infection may lead to hyperinsulinaemia, resulting in increased growth of the foetus. Alternatively, transient hyperglycaemia associated with insulin resistance might affect foetal growth. In addition, cytokines may affect beta cell function to contribute to hyperinsulinemia or may directly influence foetal growth, although nothing is yet known about the existence of such mechanisms. While metabolic responses in the mother are likely to influence the growth of the foetus, other explanations of the interaction between infections, HLA and HrBW need to be evaluated. Perhaps, there may be a direct effect of the infectious agent on the foetus. It is known that some strains of Coxsackie and echoviruses can be transmitted across the placenta, thereby infecting the foetus. It is possible that individuals with different HLA genotypes do not respond equally to infections. The divergence in HLA heterodimers encoded by different HLA genotypes, ensures that all individuals will not present the same peptides on the cell surface. It has been found that children with the high-risk genotypes HLA-DR-3 and 4 react with a stronger humoral response to infections with and after vaccinations to enterovirus, compared to children with other HLA genotypes [227]. It has also been reported that children with HLA-DQ2 without DQ8 have fewer enterovirus infections than children with both HLA-DQ2 and DQ8 [110], which therefore may make the carrier more susceptible to this kind of infection. However, in the present study, mothers of children with the HLA high-risk genotype did not report more frequent infections than other mothers. The child and not the mother was HLA typed in our investigation, but at least one of the HLA-DQ alleles of the foetus will be shared by the mother.

## Cord blood islet autoantibodies and BW

A majority of cord blood autoantibodies are transferred across the placenta from the mother and autoantibody-positivity that persists from birth is very rare [38]. It is not known

whether autoantibodies in cord blood interact with HLA genotypes, gestational infections and other risk factors for type 1 diabetes. In papers I and II we therefore analysed possible relationships between cord blood autoantibodies, HLA genotypes, BW and infections during pregnancy. We excluded mothers with diabetes, but still 3 % of the term children had autoantibodies to GAD65 or IA-2. Autoantibodies to insulin were only included in paper II, since the analyses were not completed at the time of the report of paper I.

In paper I we reported that children with autoantibodies to GAD65 in cord blood were less likely to be born with HrBW (OR=0.78, p=0.03). The relationship was independent of the HLA genotype of the child and persisted after compensation for confounding factors. The same effect was found when analysing the larger cohort of children in paper II. The presence of IA-2 or IAA in cord blood did not affect the BW of the child. However, when infections during pregnancy were taken into account, both GAD65Ab (OR=0.35, p=0.002) and IAA (OR=0.21, p=0.02) were significantly associated with decreased risk of HrBW for children born to mothers who had reported infections, while the presence of IA-2Ab did not reach statistical significance. This was also true when presence of any of the three autoantibodies (GAD65Ab, IA-2Ab or IAA) in cord blood was used as a criterion (OR=0.34, p=0.0002). There was no association between islet autoantibodies and HrBW in children of mothers without reported infections. Thus, all the reported reduced risk of HrBW in the presence of cord blood autoantibodies may be explained by gestational infections (Figure 6). We considered the possibility that infections during pregnancy could induce islet autoantibodies in the mother or child, resulting in positive cord blood autoantibodies. However, our data did not reveal an increased incidence of positive cord blood autoantibodies in children born to mothers who reported gestational infections. Therefore, infections during pregnancy do not seem to induce islet autoantibodies in mother or child.

Our data suggest a complex interaction between HLA genotypes, islet autoantibodies and rBW. Islet autoantibodies in cord blood from children of mothers reporting infections reduced the rBW regardless of the HLA genotype of the child. Interestingly, in a study of children born to mothers with type 1 diabetes, the hypothesis that IAA would increase foetal insulin and BW was tested. No such increment was found, but a non-significant trend of decreased BW with increasing IAA could be seen [228]. This is consistent with our data in this investigation of children born to non-diabetic mothers. Although we do not know the HLA genotype of the mother, it may be anticipated that she carries at least one allele that is diabetes high-risk, which is associated with islet autoantibodies in the healthy population [68]. Therefore, the reduced risk of HrBW may be due to the possibility that islet autoimmunity was already present in some mothers reporting infections. In autoantibody-positive pregnant mothers, the immune response to infections may be affected in a way that reduces rather than accelerates foetal growth.

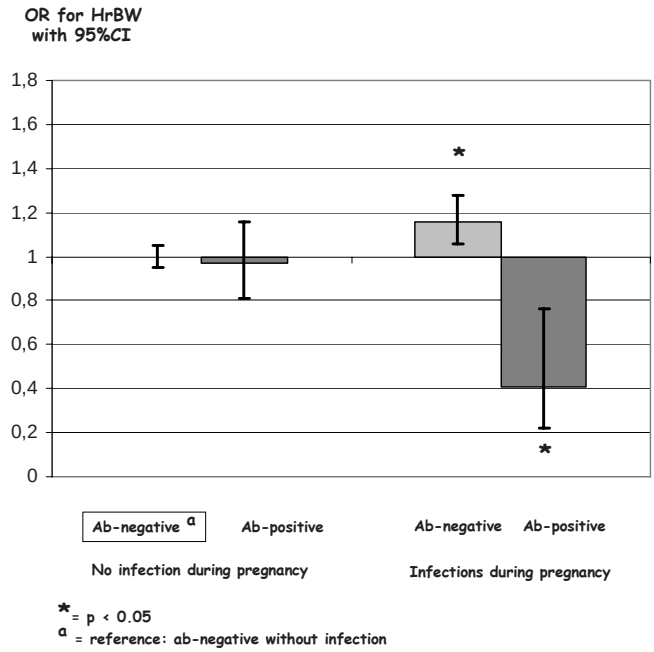



Figure 6. OR for HrBW in children with or without infections during pregnancy and with and without antibodies in cord blood. Reference: Children with no infection and negative for cord blood autoantibodies.

# Intrauterine growth, HLA and diabetes

In paper III we tested the hypothesis that children developing diabetes have an altered growth both during pregnancy and in early childhood.

We investigated intrauterine and childhood growth in all children born within the screening years of DiPiS and who had developed diabetes until the end of 2006. This population-based cohort of children developing diabetes before the age of six, represented a diabetes incidence as high as 40/100,000 children a year. They were compared to non-HLA matched as well as HLA matched controls. Since both BL, BW and childhood linear growth are strongly correlated to the height of the parents [175, 176, 229], MPH SDS was corrected for. The previously described correlation between parental height and either BW or BL was also confirmed in our study.

Children developing diabetes did not have a higher BL or BW SDS than the controls. However, when BL SDS was corrected for MPH, children developing diabetes had a higher score compared to non-HLA matched controls ($p<0.014$) or to all controls ($p<0.017$). In contrast, no significant difference was found when compared to HLA matched controls. In a multivariate analysis, we made the novel observation that BL SDS was increased in children with high-risk HLA, regardless of whether the children developed diabetes or not. However, BW SDS was not

significantly increased in children with high-risk HLA, probably due to the limited number of children in our study. Furthermore, when correcting for HLA genotypes, no significant association between either BL or BW SDS and development of diabetes was found, thus contradicting previous epidemiological studies that have reported a higher BW of children developing type 1 diabetes [82, 83, 154, 155]. Since the previous studies included children up to 15 years of age it is possible that increased BW in children developing diabetes is less common in the very young. Indeed 1/3 of our children developed diabetes before two years of age. Another reason for our failure to find a higher BW in children with diabetes could be that our matching of controls minimized the effect, since the children were matched for gender, gestational week as well as date of birth. On the other hand, when BL was corrected for MPH we found that our children developing diabetes were taller than the controls, which is consistent with the previous finding that a short BL decreased the risk for type 1 diabetes in children up to 15 years of age [79]. The fact that BL corrected for MPH was increased in the diabetes children compared to non-HLA matched controls but not to HLA matched controls could possibly be due to our limited number of HLA matched controls. Still, this is an important novel finding, especially since we found that BL was significantly associated to HLA genotypes. Thus, both the earlier observations of increased BW and BL in children developing diabetes may be due to an effect of high-risk HLA on intrauterine growth.

Together with our previous finding that infections during pregnancy interact with HLA in affecting BW, the data has led us to hypothesise that different ways of handling infections, with or without subsequent temporary hyperglycemia, may at least partly explain how HLA genotypes affect intrauterine growth. Unfortunately, the information about infections during pregnancy was incomplete in the children developing diabetes, as not all parents had filled out the 2-month questionnaire. Therefore no analysis of the effect of infections on BW and development of diabetes could be done. Future analyses with access to an increasing number of DiPiS children developing diabetes will answer this question.

## Childhood growth, HLA and diabetes

Since our initial studies showed that diabetes high-risk HLA genotypes correlate to both BW and BL SDS and also indicate that the increased risk for type 1 diabetes previously observed with high BW might be dependent on the interaction between HLA genotypes and infections during pregnancy, we also wanted to see if HLA genotypes affected postnatal growth and could be responsible for the increased linear growth previously reported in children developing diabetes.

When analysing childhood linear growth and BMI we excluded values obtained after diabetes debut. The growth up to 18 months of age was studied. Children developing diabetes before six years of age were significantly taller than expected from MPH in comparison to all controls, non-HLA matched controls as well as HLA matched controls (Figure 6). Children developing diabetes also had a higher linear growth rate than HLA matched controls at 3 ($p<0.037$), 6 ($p<0.035$) and 18 ($p<0.038$) months of age. In the children developing diabetes, we

found an age-dependent trend of increased difference between the height of the child and the expected value based on MPH with increasing age, underscoring the accelerating height development. When comparing the change of Diff MPH (Diff MPH = SDS height of child – SDS MPH) 18 months – baseline, between children developing diabetes and HLA matched controls or non-HLA matched controls, the diabetic children had gained significantly more in this value ($p<0.05$) (Figure 7).

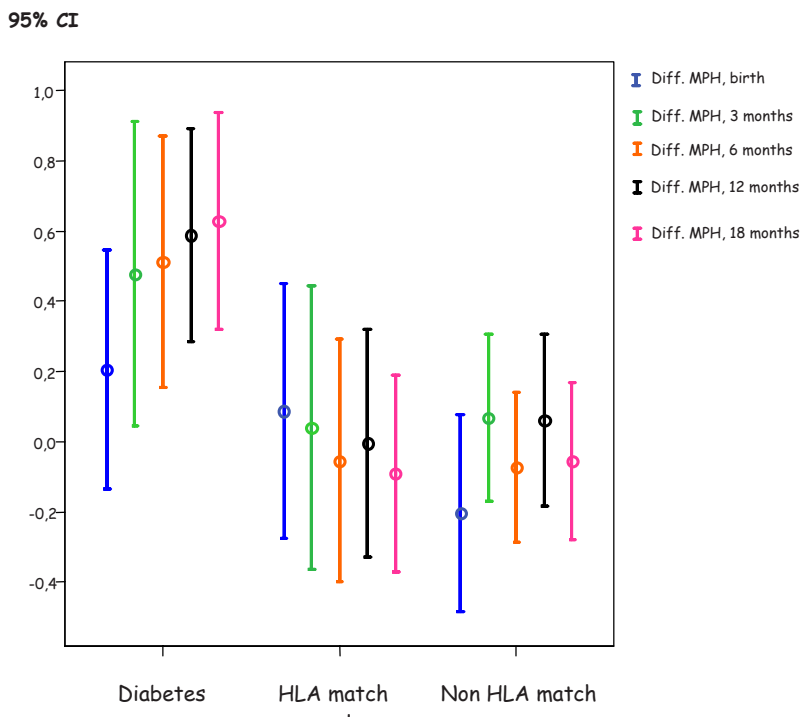



Figure 7. Height compared to MPH in children developing diabetes and in HLA matched and non-HLA matched controls.

Increased height and linear growth in childhood have been reported as risk factors for diabetes [165-167]. This was confirmed in our study. We hypothesised that HLA genotypes conferring risk of diabetes were responsible for the increased growth in children who later develop diabetes. Our data did not support this hypothesis, and rather indicate that other factors must be responsible. Since nutritional factors and insulin are the most important growth promoters in infancy, one reason for the increased growth rate in the children who later develop diabetes could be changes in the metabolism associated with the autoimmune destruction of the pancreatic beta cells. Another hypothesis is that type 1 diabetes arises due to altered eating habits in our welfare society [164, 169]. Over-nutrition may cause increased growth in infancy, since

obesity in this period of life is likely to result in increased height gain. It could possibly also result in increased insulin resistance which would stress the beta cells and either trigger the autoimmune reaction via increased apoptosis and subsequent exposure to islet autoantigens with type 1 diabetes as a result, or accelerate an already initiated autoimmune reaction [164]. This 'overload hypothesis' is also consistent with the earlier described increase in BMI [166-168, 170] and energy intake [153] in children who develop type 1 diabetes. In our cohort of children, we did not observe any significant increase in BMI SDS in children who later developed diabetes compared to non-HLA matched controls or to all controls. On the contrary, there was a significantly lower BMI at 6 months of age in children developing diabetes compared to HLA matched controls ($p<0.010$). Furthermore, the children developing diabetes had a significantly lower BMI SDS than the Swedish standard population both at 12 months ($p<0.006$) and at 18 months ($p<0.003$) of age. The lack of an increased BMI in our children could be consistent with the view that young children increase their height rather than weight in response to overfeeding. Whether the children developing type 1 diabetes in our study were overfed or not is not known. The lower BMI in these children compared to their HLA matched controls at 6 months of age, however, is inconsistent with previous findings of increased BMI and the 'overload hypothesis'.

In conclusion, HLA genotypes did not explain the previously described height gain in infancy in children developing diabetes, and we therefore hypothesise that height gain is related to the type 1 diabetes disease process.

## Parents' reactions to genetic screening

In paper IV we investigated the parents' reactions to participation in DiPiS and compared the attitudes of the mothers and fathers. When this investigation was performed, the parents were not informed about the diabetes risk of their children. Thus, this study is based on the response to participation in a screening study, but not on how parents react to the information about diabetes risk. A total of 46/62 (74 %) mothers with diabetes participated. In 2.2 % of all the participating families, a family member had diabetes (46 mothers, 89 fathers, 12 siblings). The mean age of participating mothers was higher than that of non-participating mothers and a larger proportion of both younger (<25 years) and older (>40 years) mothers were non-participants. Parents of twins and of prematurely born children were underrepresented among participating parents compared to the entire population.

Even if most parents were satisfied with the information about the study, parents in families with one family member (mother, father or child) having type 1 diabetes were dissatisfied to a larger extent ($p<0.003$). These parents also estimated the risk to their newborn child of developing diabetes to be higher than parents in families without diabetes ($p<0.001$). The mothers in these diabetes families expressed more worries (42 %) regarding the likelihood of the child developing a chronic and serious disease, than did fathers with a diabetic family member (26%) and parents in non-diabetes families (27 %).

Most parents reported that they were neither reassured nor worried when participating in DiPiS. A larger proportion of parents were reassured than the proportion of parents reporting more worries. Only 1.1 % of mothers and 1.2 % of fathers participating in the study reported increased worries, while increased worries with participation in DiPiS was reported by 2.8 % of mothers in families with a diabetic family member. However, the difference in response between families with and without diabetes was not significant. Worries about the future risk of chronic and serious disease were more often reported by parents with lower education, being born outside Sweden, being younger, having less knowledge of diabetes and ascribing diabetes risk to the child. Also mothers with diabetes in the family, single mothers and mothers who were dissatisfied with the study information expressed more worries. However, mothers with low education, younger mothers and mothers born outside Sweden did also to a higher extent answer that they were reassured by participation in DiPiS. For the fathers, a lower education and being born outside Sweden contributed to reassurance with participation, while diabetes in the family, ascribing high risk of diabetes in the child and dissatisfaction with the information about the study increased the worries.

In conclusion, taking part in DiPiS as a screening study for type 1 diabetes did not create worries in most parents, but it is important to note that the parents were not informed about the child's risk for type 1 diabetes when the investigation was performed. The lack of feedback may explain why a majority of the parents reported that they were not affected at all by participation in the study. The finding that 1.1 % of all parents nevertheless reported more worries is consistent with another study in which the screening result was not given to the parents if they did not request the information [209]. Even in this study mothers with diabetes in the family reported a little more worry. Consistently, other studies of the general population in which information about the risk was given to the parents, suggest that screening and notification of high type 1 diabetes risk in the newborn child may not result in increased levels of parental stress [208]. This also seems to be true in studies based on type 1 diabetes families, where testing for autoantibodies and information about the risk reduced anxiety [230]. However, with information of positivity for islet cell autoantibodies, increased anxiety was seen, which emphasises the importance of providing accurate risk information and counselling.

Proper information about studies of this kind is important to prevent worries, since dissatisfaction with the information was associated with more worries in both fathers and mothers. The majority of the parents were satisfied with our study information, but fathers tended to be more unsatisfied. To be informed about DiPiS the father had to accompany the mother to the Maternity Health Care Clinics where the information was given, or receive information indirectly from the mother, which probably explains this discrepancy in answers.

In conclusion, screening for type 1 diabetes in newborns does not cause more worries in most parents and proper information helps in reassuring most parents. This study and other studies also suggest that feedback to the parents regarding the diabetes risk of the child may lessen the parents' worries about their child. More studies of this ethical issue in screening studies in the general population are needed.

# Possible benefits of screening

As of December 2006 a total of 60 children born within the screening years of DiPiS had developed type 1 diabetes. A vast majority of these children had high-risk HLA genotypes for type 1 diabetes, but 5 children had genotypes normally not conferring type 1 diabetes risk (Figure 8). DiPiS was designed to follow high-risk children from 2 years of age. Unfortunately, we did not have ethical permission to inform the parents about the diabetes risk of their children from the study start. After approval from The Ethics Committee, the parents of children born from 2002 were informed about the estimated risk when the child was 2 years of age, if they sent in an agreement to receive information. In combination with the somewhat surprisingly high number of 19 children developing diabetes before 2 years of age, this makes an analysis of the benefits of screening and information about risk in the study difficult. Only parents of 5/60 children developing diabetes until the end of 2006 had been informed about type 1 diabetes risk before onset of disease and these children were diagnosed at an age above 2 years.

Of the 19 children developing diabetes before 2 years of age, 11 (58 %) had DKA at onset of diabetes and 2 children (11 %) had severe DKA (pH<7.10). This high number of ketoacidosis in young children is consistent with other studies, where children diagnosed with type 1 diabetes before 2 years of age were reported to have DKA in 40-53 % of the cases and severe DKA in 10% [62, 204]. In DiPiS, no difference was seen in frequency of DKA at diagnosis between participating and non-participating children diagnosed with diabetes before 2 years of age. DKA at onset of disease was present in only 5/41 (12 %) children older than 2 years. Of these, 2 were participating and 3 non-participating children. However, none of the parents of children with DKA at diagnosis had been informed about diabetes risk or positive autoantibodies prior to the diagnosis of diabetes.

It could be assumed that the parents, if informed about the risk of type 1 diabetes, would be more observant on symptoms and signs of diabetes even for the young children, thereby potentially preventing serious DKA. To date, none of the 5 children in DiPiS with high-risk HLA genotypes and 2-3 autoantibodies, followed every 3 months in DiPiS, have had DKA at onset of disease. In fact, 3 children have been diagnosed without obvious diabetes symptoms, at a regular visit in DiPiS. For those children the participation in DiPiS may have been of benefit, since an early diagnosis with mild or no symptoms is associated with significant residual capacity of the beta cells to produce insulin and a longer 'honey-moon' and higher levels of C-peptide [196].

Whether DKA can be prevented by information about risk and by following islet cell autoantibodies will be revealed in presently ongoing studies. One of these is TEDDY, a multicentre study following children with high genetic risk for type 1 diabetes and screening for autoantibodies where parents are informed about diabetes risk already when their child is 3 months of age.

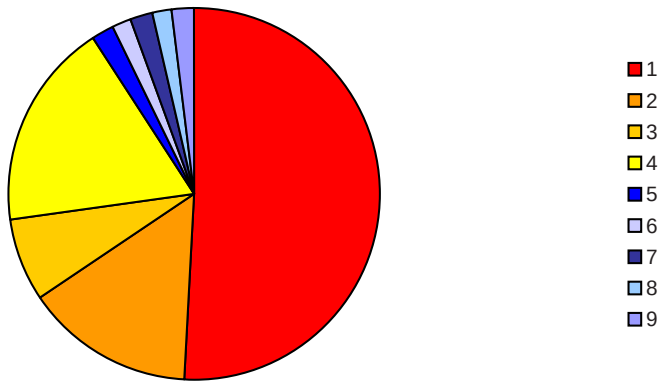
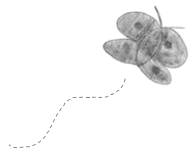



Figure 8
HLA-DQA1*-B1* genotypes in the 55 HLA-typed children developing diabetes up until December 2006, born within the screening years of Diabetes Prediction in Skåne (DiPiS) study. High or moderate type 1 diabetes risk genotypes are indicated with red and yellow while neutral or low risk genotypes are blueish.
1) HLA-DQA1*-B1* 0501-0201/0301-0302 (DQ2/8) n=28 (52%); 2) DQA1*-B1* 0301-0302/X-X n=8 (15%); 3) DQA1*-B1* 0301-0302/0102-0604 n=4 (8%); 4) DQA1*-B1* 0501-0201/X-X n=10 (18%). Only 5 children (9 %) had neutral or low risk genotypes; 5) HLA-DQA1* 0201-0201/X-X n=1; 6) DQA1*0201-0201/0301-0302 n=1; 7) DQA1* 0301-0301/0301-0302 n=1; 8) DQA1*0301-0301/0501-0201 n=1 and 9) DQA1*0103-0603/0301-0302 n=1.X is not DQB1 02, 0301, 0302, 0602, 0603 or 0604.



# Chapter 6
# GENERAL DISCUSSION

In this thesis the relationships between gestational and post-natal risk factors for type 1 diabetes have been dissected and some previously unknown interactions have been reported. This gives a new perspective on the still unresolved issues of the aetiology and pathogenesis of type 1 diabetes. The studies have also shed new light on issues regarding benefits and drawbacks of screening and prediction of type 1 diabetes in children in the general population.

Our novel result that diabetes high-risk HLA genotypes may affect intrauterine growth has been recently confirmed also in children born to mothers with type 1 diabetes [231], although another study could not repeat the finding [232]. Our additional observation of an association between HLA genotypes and BL strengthens the suggestion that HLA influences growth *in utero*. We could also show an association between reported infections during pregnancy and increased BW of the child. The association was particularly strong for children with high-risk HLA genotypes. We therefore hypothesise that the association between HLA genotypes, gestational infections and high BW is due to different ways of handling infections in either the mother or the child, depending on the HLA genotypes. Diabetes high-risk HLA genotypes may be associated with stronger immunological responses to gestational infections. This may result in transient hyperglycemia or increased insulin resistance compensated for by higher levels of insulin. The finding that intrauterine infections interact with HLA genotypes and cord blood islet cell autoantibodies to affect intrauterine growth is also of interest, since infections during pregnancy have been reported as a risk factor for type 1 diabetes.

We find it reasonable to assume that previous observations of an association between high BW and type 1 diabetes are secondary to an interaction between HLA genotypes and infections during pregnancy affecting intrauterine growth. Thus, most likely, high BW *per se* does not primarily increase the risk of type 1 diabetes. Further studies will be needed to clarify the importance of the number of infections during pregnancy and the relative impact of specific infectious agents in conferring the effects on the rBW. This needs to be put in relation to the HLA genotype of both the mother and the child. Also, the future risk of diabetes for the children

born to mothers reporting infections during pregnancy, and born with a type 1 diabetes high-risk HLA genotype and HrBW, is still unknown. The number of children, participating and followed in DiPiS, who have developed diabetes is still too small to analyse these relationships.

Yet, children developing diabetes have an increased linear growth in childhood, regardless of their HLA genotype, indicating that factors other than HLA are important for this increment in height. Whether metabolic changes in the early autoimmune destruction of the beta cells are responsible for this fact or if it is due to an increased energy intake accompanied by altered eating habits remains to be determined. It is also not known if this increase in childhood linear growth is associated with gestational or childhood infections, since we still do not have a long enough follow-up in the DiPiS study to elucidate this kind of difference.

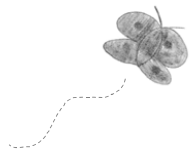
Also the role of islet cell autoantibodies in cord blood on BW, as well as for diabetes risk, remains unclear. A decreased risk of developing diabetes with cord blood autoatibodies in offspring of type 1 diabetes mothers has been reported [39], suggesting that autoantibody-positivity at birth may protect the child. If this is due to 'immunisation' in foetal life or other effects is still not known. In contrast, it was recently reported that type 1 diabetes children who were diagnosed without islet autoantibodies more often had autoantibodies in cord blood at birth [35], suggesting that an autoimmune process early in life that later has decreased in activity may predispose for type 1 diabetes later in life. In our study we found that islet cell autoantibodies in cord blood decreased the risk of HrBW in the child only if the mother reported infections during pregnancy. This effect was seen independently of the HLA genotype of the child. Thus, there is a complicated interaction between HLA genotypes and islet cell autoantibodies and infections during pregnancy in affecting the growth of the foetus, which needs to be further studied to clarify the significance of these effects for development of type 1 diabetes in the child.

Since environmental risk factors are important for the development of type 1 diabetes and probably explain the increasing incidence of the disease, risk factor analysis is valuable for the understanding of the disease. When we identify the factors that trigger or accelerate the disease, a possibility for prevention may arise. Therefore, even small pieces in the puzzle of diabetes aetiology may be of importance, even if more research is needed to confirm and explain the mechanisms of these findings.

Since only a minor portion of children developing type 1 diabetes are first-degree relatives of patients with the disease, population-based investigations of triggering and accelerating factors as well as the natural history of the disease are important. Furthermore, the ethical dilemmas of screening for a disease with no current prevention and a small risk of disease progression are not easy to resolve. However, in our study we did not find that screening in the population resulted in increased anxiety. This has to be further analysed during the follow-up of DiPiS, since the information of diabetes risk in the children either may increase or decrease the parents' anxiety.

At present, the only benefit of predicting type 1 diabetes is the important possibility of early diagnosis with remaining beta cell activity and less risk of life threatening DKA at clinical onset of disease. With increasing knowledge about factors that trigger and accelerate type 1 diabetes, the disease may be preventable by interventions against these risk factors, for example through dietary modifications such as high dose omega-3 fatty acids, vitamin D supplementation

or the use of hydrolysed formula. Still, it may be impossible to eliminate some risk factors. Therefore trials with medications modulating the immune system are also important. Previous immune interventions have had too serious side effects to outweigh the scarce positive effects. However, there are some promising ongoing trials with autoantigen therapies, of which vaccination with GAD65 has shown promising results initially, with preservation of residual insulin secretion in children with newly diagnosed type 1 diabetes and without side effects [196]. Further studies are needed, but if this vaccination can preserve beta cells it might even prevent disease in children with ongoing beta cell destruction who have not yet developed clinical diabetes. If we then could foresee which children will develop diabetes, we would also have the possibility to prevent this serious, demanding and devastating autoimmune disease.



# Chapter 7
# CONCLUSIONS

1) Type 1 diabetes high-risk HLA genotypes increase the SDS of BW and BL in the newborn child, probably by affecting intrauterine growth of the foetus.

2) Infections during pregnancy increase the rBW in the newborn child, probably due to an interaction between infections and HLA genotypes affecting intrauterine growth of the foetus.

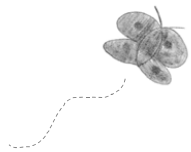
3) Infections during pregnancy and cord blood islet cell autoantibodies interact with intrauterine growth, resulting in decreased risk of HrBW in the child, independent of HLA genotypes.

4) The effect of infections during pregnancy and HLA-genotypes on intrauterine growth may explain the previously described increase in BW in children with type 1 diabetes.

5) Linear growth corrected for MPH is increased during the first 18 months of age in children developing diabetes before 6 years of age, independent of HLA genotypes.

6) Prospective screening for type 1 diabetes in a population-based study, where no information on risk is given to the parents, does not cause anxiety in most parents. Reactions of mothers and fathers differ and proper information is of importance to reduce the risk of anxiety.

7) Screening for type 1 diabetes may be of benefit to the child in terms of preventing DKA at the onset of diabetes, but further studies are needed to confirm this observation.



# Chapter 8
# SWEDISH SUMMARY

Typ 1 diabetes är en av de vanligaste kroniska autoimmuna sjukdomarna hos barn. Sjukdomen kräver livslång insulinbehandling och medför enorma påfrestningar i vardagslivet både för barnet och för hela familjen, samt en hög risk för allvarliga långtidskomplikationer. För att utveckla typ 1 diabetes behövs dels en genetisk (ärftlig) känslighet, så kallad predisposition, och dels utlösande och påskyndande faktorer i omgivningen. Innan sjukdomen bryter ut pågår en obemärkt autoimmun process, då det egna immunförsvaret bryter ner de insulinproducerande betacellerna i bukspottkörteln. Vilken eller vilka omgivningsfaktorer som utlöser och påskyndar denna process hos genetiskt känsliga individer är fortfarande oklart. Genom att undersöka genetisk känslighet och genom att mäta de antikroppsmarkörer som är ett mått på den pågående autoimmuna processen, kan sjukdomen till viss del förutsägas (predikteras).

I den här avhandlingen sammanfattar jag den kunskap som hittills finns om riskfaktorer och prediktion av typ 1 diabetes hos barn samt beskriver våra fynd av tidigare okända samband mellan några av dessa riskfaktorer. Artiklarna i avhandlingen baseras på undersökningar i den så kallade Diabetes Prediktion i Skåne (DiPiS) studien. I denna studie har alla barn födda i Skåne under september 2000 tom augusti 2004 erbjudits deltaga. Över 35000 barn har undersökts för genetisk risk för typ 1 diabetes i navelsträngsblod. En grupp barn med ökad risk för sjukdomen följs från 2 års ålder med årliga blodprov och frågeformulär. Barn som utvecklar autoantikroppar som mått på att en autoimmun process mot de insulinproducerande betacellerna är pågående, följs var 3:e månad med blodprov och läkarkontakt.

Hög födelsevikt har tidigare beskrivits som riskfaktor för typ 1 diabetes. I **artikel I** beskriver vi samband mellan HLA-gener, som står för den största delen av den genetiska risken för typ 1 diabetes, och födelsevikt. Barn med HLA-gener med hög risk för typ 1 diabetes visar sig också ha en ökad risk för hög födelsevikt. Sambandet mellan HLA-gener med hög risk för typ 1 diabetes och födelsevikt kan förklara den tidigare beskrivna iakttagelsen att barn med diabetes har ökad födelsevikt. Detta kan i sin tur innebära att den ökade födelsevikten hos barn som utvecklar

diabetes är sekundär till sambandet mellan HLA-gener och födelsevikt, och inte i sig ger en ökad risk för diabetes.

Hur HLA-gener skulle kunna påverka födelsevikten är inte klart. Dessa gener kodar för proteinkomplex som är viktiga i vårt immunförsvar, genom att hjälpa till att ”presentera” olika så kallade antigen för de celler som ingår i immunförsvaret. På så sätt kan en immunreaktion komma igång för att t ex bekämpa virus och bakterier. Eftersom olika HLA-gener kodar för olika sådana proteinkomplex, kan immunreaktionen bli olika stark. Om en kvinna får en infektion under graviditeten kan hennes HLA-gener ha betydelse för hur kraftigt infektionen bekämpas av immunförsvaret. Detta kan i sin tur möjligen påverka fostret.

I **artikel II** beskriver vi hur infektioner under graviditet påverkar födelsevikten. Barn födda till mammor som rapporterat att de haft infektioner med feber, diarré eller kräkningar under graviditeten visar sig ha en ökad risk att födas med hög födelsevikt. Sambandet mellan HLA-gener med hög risk för typ 1 diabetes och födelsevikt förstärks när mamman har rapporterat infektioner under graviditeten. Infektioner kan delvis, men inte helt, förklara varför barn med HLA-gener som medför hög risk för diabetes har ökad risk för hög födelsevikt.

Eftersom barnet ärver hälften av sina HLA-gener från mamman, har mamman minst en HLA-allel (del av HLA-gen) som är identisk med barnets. Om barnet har två alleler med hög risk för diabetes, kommer således mamman att ha minst en högrisk-allel. Dessa HLA-alleler har tidigare visat sig ge en kraftigare immunreaktion vid vissa infektioner. Vid infektioner och immunförsvarspådrag blir känsligheten för insulin nedsatt och en kraftig reaktion kan kanske minska känsligheten för insulin ytterligare. För att kompensera för detta måste betacellerna i bukspottkörteln utsöndra mer insulin, annars stiger blodsockret. Tillväxten hos ett foster är främst beroende av näringstillförsel via moderkakan och insulin. Ett högt blodsocker hos mamman, eller ökade insulinnivåer, kan stimulera fostrets tillväxt. Dessa mekanismer kan eventuellt förklara sambanden mellan HLA-gener med hög risk för diabetes, infektioner och födelsevikt.

I **artikel III** beskriver vi tillväxten under de första 18 månaderna i livet hos 58 barn som utvecklat diabetes före 6 års ålder. Alla dessa barn är födda under åren för screeningen i DiPiS. Vi jämför barnens tillväxt med kontrollbarn i DiPiS, matchade för kön, födelsedag och graviditetsvecka. 1/3 av barnen är också matchade för HLA-genotyp. Tillväxten undersöks via tillväxtkurvor inhämtade från barnavårdscentraler och korreleras också till föräldrarnas längder. Det visar sig att barnen som utvecklar diabetes inte har någon ökad födelsevikt. Födelselängden är däremot ökad hos barn som utvecklar diabetes jämfört med icke HLA-matchade kontroller, men inte jämfört med HLA-matchade kontroller. Födelselängden visar sig vara korrelerad till HLA-genotyp, vilket kan förklara att barn som utvecklar diabetes är längre än icke HLA-matchade kontrollbarn. Barnen som senare utvecklar diabetes växer däremot mer på längden under de första 18 månaderna än både HLA-matchade och icke HLA-matchade kontroller, efter att längderna är korrigerade för föräldrarnas längd. Barn som utvecklar diabetes har däremot inte ökad vikt eller ökat body mass index (BMI) jämfört med kontroller.

Varför barn växer mer på längden innan de får diabetes vet vi inte. En orsak kan vara att barn med en ännu oupptäckt autoimmun reaktion, som så småningom kommer att leda till

diabetes, har en ändrad ämnesomsättning. Ett ändrat mönster i insulinsekretionen, den autoimmuna processen i sig eller en oupptäckt långvarig virusinfektion skulle kunna påverka tillväxten tidigt i livet. Med detta resonemang skulle den ökade tillväxten vara sekundär till den autoimmuna processen. En annan orsak skulle kunna vara att dessa barn har ett högre intag av energi eller näringsämnen, vilket orsakar en ökad tillväxt. Tillväxten i sig skulle då kunna stressa betacellerna och trigga igång den autoimmuna reaktion som leder till diabetes. Fortsatta studier krävs för att ta reda på dessa orsakssamband.

I **artikel IV** beskriver vi föräldrarnas reaktioner på att deltaga i DiPiS. Undersökningen baserar sig på föräldrarnas svar på frågeformulär utskickade under den första tiden av screeningen i DiPiS. Vid denna tidpunkt hade föräldrarna inte informerats om barnets risk att få diabetes. Undersökningen visar att majoriteten föräldrar inte oroas av att deltaga i denna screening-studie för diabetes, men att mammor i familjer där någon familjemedlem redan har diabetes är något mer oroade. Undersökningen visar också att pappor och mammor reagerar olika på att deras barn deltar i DiPiS och att fullgod information om studien minskar oron. Fortsatta studier av föräldrareaktioner är viktiga efter det att föräldrarna fått information om barnens risk att få diabetes.

DiPiS är en av flera pågående longitudinella studier där barn med hög risk för diabetes följs från födseln. Dessa studier kommer förhoppningsvis att bidraga med kunskap om varför barn utvecklar typ 1 diabetes och hur den autoimmuna processen fortgår. Stora, populationsbaserade studier behövs för att ta reda på vilka faktorer som är inblandade i att trigga igång och påskynda den autoimmuna processen. I den här avhandlingen har jag beskrivit några hittills okända samband mellan möjliga riskfaktorer för diabetes. Dessa fynd är endast små pusselbitar, men kan förhoppningsvis bidraga till ökad kunskap om riskfaktorer och sjukdomsförlopp.

Vad har då barnen och föräldrarna för nytta av att deltaga i populationsbaserade screening-studier som DiPiS? För närvarande finns inget säkert sätt att bromsa eller att stoppa sjukdomsutvecklingen. Lovande försök har utförts med bland annat vaccin mot en av diabetesautoantikropparna, GAD65, på barn och vuxna med nyligen diagnostiserad typ 1 diabetes. Detta vaccin har dock ännu inte provats på barn som inte utvecklat diabetes men som har en pågående autoimmun process i sin bukspottkörtel. Fram till dess att säkra och fungerande medel att stoppa den process som leder till att typ 1 diabetes utvecklas finns att tillgå, får vi nöja oss med att följa dessa barn. Även detta kan vara av godo för barn och föräldrar. Genom att diagnostisera diabetes i ett tidigt förlopp ökar chansen för en lång period med lägre behov av insulin och en mer lättstyrd diabetes. Mer än 50 % av barn som utvecklar diabetes före 2 års ålder är vid diabetesinsjuknandet sura i blodet - de har en så kallad ketoacidos. Ketoacidos är ett allvarligt sjukdomstillstånd och kan i vissa fall vara direkt livshotande. Genom att informera föräldrarna om den ökade risken för diabetes och genom att följa barn som utvecklar diabetesantikroppar, finns det möjlighet undvika att barnet insjuknar i en ketoacidos. Detta är till godo för både barn och föräldrar, liksom för samhället i stort.

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