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# Triggers of autoimmunity

Studies on gestational events



**LUND**  
**UNIVERSITY**  
Faculty of Medicine

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2011

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# Triggers of autoimmunity

Studies on gestational events



**LUND**  
**UNIVERSITY**  
Faculty of Medicine

## Doctoral dissertation

By due permission of the Faculty of Medicine, Lund University, Sweden  
to be defended in "Jubileumsaulan", entrance 59, Malmö University  
Hospital at 9.00 am, Friday the 3<sup>rd</sup> of June 2011

Faculty opponent: Lars Christian Stene  
Oslo, Norway



“All that is necessary for the triumph of evil is for good  
men to do nothing”  
- Edmund Burke



# Abstract

## Objective

The primary aim of this thesis was to test whether gestational exposure to environmental factors may induce islet autoimmunity. The second aim was to determine to what extent the exposure to viruses or other environmental factors is a risk factor for type 1 diabetes in the offspring. As children with type 1 diabetes have a higher risk of developing celiac disease (CD), an additional aim was to determine whether markers of possible infections during early pregnancy was associated with development of tissue transglutaminase (tTG) autoantibodies or CD in the offspring. These aims have been summarized in four independent studies.

## Study population and Methods

The Diabetes Prediction in Skåne (DiPiS) is a population based study where blood samples were obtained at the time of delivery between September 2000 and August 2004 from all mothers in this region (Skåne). The blood samples were analyzed for HLA-DQ alleles and islet autoantibodies.

The prospective cohort study Celiac Disease Prediction in Skåne (CiPiS) is part of the DiPiS study, which aims to determine the etiology indicators of celiac disease (CD) in newborn children. Children with HLA-risk alleles associated with CD were screened for tissue transglutaminase autoantibodies (tTGA). CD was established by intestinal biopsy in children with confirmed tTGA.

Early pregnancy serum samples (gestational week 10-16) were collected from the Southern Sweden Microbiological biobank (SSM-Biobank). This biobank contains more than 120,000 stored plasma samples that have been obtained between 1986 and 2009 from all pregnant mothers at their first visit to the Maternity Care Center.

By combining the SSM-Biobank with DiPiS and CiPiS, a total of 24,000 mothers were identified. All mothers included in this thesis (n=1,748) were analyzed for HLA genotype, islet autoantibodies (GADA, IA-2A and IAA in both early pregnancy samples and at delivery), nine different cytokines, one chemokine (early pregnancy samples), IgM class enterovirus (EV) antibodies and EV-RNA (early pregnancy samples).



## Study I-IV

Study I: The objective of our first study was to determine seroconversion to islet autoantibodies in non-diabetic mothers during pregnancy. This was achieved by analyzing end point titers of GADA, IA-2A and IAA in both early pregnancy samples and samples at delivery. Mothers positive for GADA (92%), IA-2A (84%) or IAA (65%) at delivery had increased titers already in early pregnancy. Titers declined for GADA ( $p<0.0001$ ), IA-2A ( $p<0.0001$ ) and IAA ( $p<0.0001$ ). Seroconversion during pregnancy was observed for GADA in 10 (8%), IA-2A in 3 (16%) and IAA in 37 (35%) mothers.

Study II: In this study, we collected early pregnancy serum samples from mothers who later gave birth to children who developed high titers of tTGA or confirmed CD. We then measured an array of Th1/Th2 cytokines (nine cytokines and one chemokine) with the aim of investigating whether CD is triggered already in utero as denoted by quantitative changes in the mother's cytokine profile. We observed that levels of seven out of ten cytokines were significantly increased in mothers who gave birth to children with CD when compared to controls.

Study III: As studies have shown that unbalanced gestational cytokine profiles have been associated with maternal autoimmune disease, preeclampsia, and recurrent spontaneous abortions we analyzed in the present study cytokines in serum samples collected during early pregnancy from mothers who gave birth to children developing multiple, persistent islet autoantibodies, type 1 diabetes, or both, before seven years of age. We found that IFN- $\gamma$  ( $p=0.02$ ) and IL-1 $\beta$  ( $p=0.04$ ) were elevated in the index mothers. All cytokines except IL-4 were highly correlated ( $p<0.0001$ ).

Study IV: Gestational EV infections have been associated with risk HLA-DR as well as with type 1 diabetes in the child. We analyzed enterovirus RNA (EV-RNA) and IgM (EV-IgM) in relation to type 1 diabetes HLA-DQ risk genotypes and to islet autoantibodies in non-diabetic mothers studied both in early pregnancy and at delivery. EV-IgM, but not EV-RNA was detected during early pregnancy in 12% (44/365) islet autoantibody positive mothers compared to 11% (156/1457) of the controls ( $p=n.s.$ ). In early pregnancy, mothers with HLA-DQ 2/2 or 2/X genotypes showed, in adjusted logistic regression, an increased risk for islet autoantibodies (OR 1.85, 95% CI 1.34-2.54;  $p=0.001$ ). EV-IgM was not associated with HLA-DQ in early pregnancy. However, after adjusting for parity, maternal age, year of birth and season of early pregnancy, early pregnancy EV-IgM combined with DQ2/2 or 2/X increased the risk for the mother to be positive for islet autoantibodies at delivery (OR 3.10, 95% CI 1.35-7.15;  $p=0.008$ ).

## Conclusion

From these studies we conclude that pregnant non-diabetic mothers with islet autoantibodies at delivery had significantly higher titers of autoantibodies during early pregnancy than at delivery. As the statistical power in the seroconverting mothers was

insufficient, further studies are needed to determine if the risk for type 1 diabetes in the offspring differs between mothers who already had increased titers of islet autoantibodies during early pregnancy or acquired them during pregnancy. Moreover, we conclude that a shift in the Th1/Th2 mediated cytokine pattern during early pregnancy, possibly caused by viral infection may be associated with CD in the offspring. Furthermore, the results from study III suggest that increased levels of IFN- $\gamma$  and possibly IL-2 during early pregnancy was associated with an increased risk for multiple, persistent islet autoantibodies, type 1 diabetes, or both, before seven years of age in the offspring. Our final study concludes that EV-IgM in early pregnancy increased the risk for islet autoantibodies at delivery in non-diabetic mothers who carry HLA-DQ 2/2 or 2/X type 1 diabetes risk genotypes.



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# List of publications included in this thesis

- I. Lindehammer SR, Hansson I, Midberg B, Ivarsson SA, Lynch KF, Dillner J, Lernmark A: Seroconversion to islet autoantibodies between early pregnancy and delivery in non-diabetic mothers. *J Reprod Immunol*; 2011. 88:72-79.
- II. Sabina Rešić Lindehammer, Sara Björck, Kristian Lynch, Charlotte Brundin, Karel Maršál, Daniel Agardh and Malin Fex: Early human pregnancy serum cytokine levels predict autoimmunity in offspring. *Autoimmunity*. Accepted for publication 2010-12-17
- III. Sabina Rešić Lindehammer, Malin Fex, Ida Hansson, Karel Maršál Marlena Maziarz, Åke Lernmark: Early pregnancy cytokines in mothers to children developing multiple, persistent islet autoantibodies or type 1 diabetes before seven years of age. Resubmitted in *American journal of reproductive immunology*.
- IV. Sabina Rešić Lindehammer, Maarit Oikarinen, Hanna Honkanen, Kristian F. Lynch, Ida Hansson, Karel Maršál, Heikki Hyöty and Åke Lernmark: Enterovirus IgM and seroconversion of islet autoantibodies. In manuscript.





# Abbreviations

APC	Antigen Presenting Cell
BB-rat	BioBreeding rat
BOX	Bart's-Oxford
CAV	Coxsackievirus A
CBV	Coxsackievirus B
CD	Celiac Disease
CiPiS	Celiac Prediction in Skåne
DASP	Diabetes Antibody Standardization Program
DIASY	Diabetes Autoimmunity Study in the Young
DiMe	Childhood Diabetes in Finland
DiPiS	Diabetes Prediction in Skåne
DIPP	Diabetes Prediction and Prevention Study
DISS	Diabetes Incidence Study in Sweden
ELISA	Enzyme-linked Immunosorbent Assay
EV	Enterovirus
GABA	Gamma-aminobutyric Acid
GADA	Glutamic Acid Decarboxylase Autoantibodies
GAD65	Glutamic Acid Decarboxylase 65
HLA	Human Leukocyte Antigen
IAA	Insulin Autoantibodies
IA-2A	Insulinoma Antigen-2 Autoantibodies
ICA	Islet Cell Antibodies
IFN- $\gamma$	Interferon-gamma

IL	Interleukin
IQR	Interquartile Range
MHC	Major Histocompatibility Complex
MMR	Measles–Mumps–Rubella
MS	Multiple Sclerosis
NO	Nitric oxide
NOD mouse	Non-obese diabetic mouse
PCR	Polymerase Chain Reaction
PTP	Protein Tyrosine Phosphatase
RIA	Radioimmunoassay
RT-PCR	Real Time- Polymerase Chain Reaction
SSM-biobank	Southern Sweden Microbiological biobank
TCR	T-cell Receptors
TNF	Tumor necrosis factor
TRIGR	Trial to Reduce IDDM in the Genetically at Risk
tTG	Tissue Transglutaminase

# Chapter 1

## Background

### The History of Diabetes

The earliest known record of diabetes dates as far back as 1552 BC. A 3rd Dynasty Egyptian papyrus mentions physician Hesy-Ra's recognition of the symptom polyuria (frequent urination) <sup>1</sup>. In the first century AD Aretaeus of Capadokia, a Greek physician described the same affliction and coined the term "diabetes", Greek word for "siphon." Eugene J Leopold portrays Aretaeus' diagnosis in his text from 1930: "Diabetes is an awkward affection melting down the flesh and limbs into the urine.... The patients never stop making water.... Life is short and painful.... They are affected with nausea, restlessness and a burning thirst and at no distant term they expire" <sup>2</sup>. In 1684 a London physician, Dr. Thomas Willis, described that the urine in patients as "wonderfully sweet as if imbued with honey or sugar" <sup>1</sup>. If it had a sweet taste he would diagnose them with diabetes mellitus – mellitus, referring to its sweetness, the Latin word for honey. The German medical student, Paul Langerhans, defended his thesis entitled "Beitrage zur mikroskopischen Anatomie der Bauchspeicheldruse" (Contributions to the microscopic anatomy of the pancreas) publicly on the 18<sup>th</sup> of February 1869. In his thesis he described the islands of clear cells throughout the gland, staining differently than the surrounding tissue and admitted that he did not know the function of these special cells <sup>3</sup>. It was not until 1893 that Gustave-Edouard Laguesse, a French pathologist from Lille, first postulated that these cells might produce an internal secretion and named them "les ilots de Langerhans" (Islets of Langerhans) in honor of their discoverer Paul Langerhans <sup>4</sup>. Laguesse postulated that the Islets of Langerhans produced secretions that played a regulatory role in digestion. Thirty years later the discovery of insulin would confirm Laguesse's postulation concerning cell secreted hormones and their role in digestion. Prior to the use of insulin, children who suffered from type 1 diabetes survived between one month to two years, with reduced diet being the only aspect employed. Before the 1900s the doctors did more harm than good including treatments such as bleedings and doping. Until the twentieth century treatments included use of opium, supplementary meals to compensate the loss of nourishment through urine and even ideas involving increased sugar consumption <sup>5</sup>. This was followed by an introduction of the opposite. The American doctor Frederick Madison Allen initiated a low-calorie diet of as little as 450 calories a day to his patients. He considered that less food would lessen the body's damage given at the time that the body of a diabetic patient was unable to process food. Michael Bliss describes it visibly in his book "The discovery of Insulin" <sup>5</sup>. "Food and drink no longer mattered often could not be taken. A restless drowsiness shaded

into semi-consciousness. As the lungs heaved desperately to expel carbonic acid, the dying diabetic took huge gasps of air to try to increase his capacity. "Air hunger" the doctors called it, and the whole process was sometimes described as "internal suffocation". The gasping and sighing and sweet smell lingered on as the unconsciousness became a deep diabetic coma. At that point the family could make its arrangements with the undertaker, for within a few hours death would end the suffering" <sup>5</sup>. Insulin was named already at the beginning of the 20<sup>th</sup> century (Eugene Lindsay Opie 1901) as the factor produced by the islets of Langerhans or "insula" (Latin). However, it would take nearly 20 years before insulin was discovered. Frederick Grant Banting, a battalion medical officer returning from France in 1917 got a part-time job in Ontario with Dr F R Miller as a demonstrator in surgery and anatomy. On October 30, 1920, Banting was preparing a seminar on carbohydrate metabolism. After reading the article "Relation of the Islets of Langerhans to Diabetes with Reference to Cases of Pancreatic Lithiasis" by Moses Barron, Banting writes on a piece of paper, "Diabetes – Ligate pancreatic ducts of dog. Keep dogs alive till acini degenerate leaving Islets. Try to isolate the internal secretions of these to relieve glycosurea" (Banting's original spelling). On Dr Millers demand Banting then visited Professor John James Rikard Macleod, well known for his expertise in carbohydrate metabolism. Professor McLeod then offered Banting's students the opportunity to pursue material for their master's thesis. Charles Herbert Best won the toss of a coin and started in May the same year <sup>6</sup>. In their work they applied Hedon's two-stage procedure<sup>1</sup> and prepared duct-ligated dogs. By the end of June there was no success. All dogs died, either of infection or bleeding. On Saturday July 30 1921, they made an extract from the shriveled pancreas of the original duct-ligated dog (animal number 391). They injected the filtrate into a white terrier (animal number 410). In 1 hour the blood sugar level dropped from 0.20g% to 0.12g%. They called this first extract "inselit" (reviewed in <sup>6</sup>). In December 1921 they sent in their first paper "The internal secretion of the pancreas" to the journal of laboratory and clinical medicine. The paper was accepted for publication in February 1922 <sup>7</sup>. After continuously modifying their method, changing filters to sterilize their extracts, using other animal extracts such as adult beef pancreas, they continued to prolong the lives of their dogs. The longevity experiment of the dog Marjorie gave Banting the opportunity to pressure McLeod to go to clinical trials. On December 2 in 1921, Leonard Thompson, a 12 year old boy, was admitted to Toronto General Hospital. He had been diabetic for 2 ½ years and was on Allens low-calorie diet in the terminal stage. He received his first injection in on January 11, 1922. Young Leonard Thompson showed signs of improved health

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<sup>1</sup> The first stage in partial pancreatectomy leaves only a small part of the pancreas, being nourished by the body's own blood supply and the drainage of the pancreatic juice into the duodenum completely cut off. At this stage, the dog does not develop diabetes. After one week, an additional surgery removes the last part of the pancreas and the animal immediately develops diabetes.

and went on to live thirteen more years taking doses of insulin. He died of pneumonia at age 27. 14 year old Elizabeth Hughes Gossett, a diabetic daughter of a US Secretary of State weighed 52 pounds when she came to Toronto in August 1922. Elizabeth Gossett received over 42,000 insulin shots during her lifetime and lived to the age of 73. The Nobel Prize in Physiology or Medicine 1923 was awarded jointly to Frederick Grant Banting and John James Rickard Macleod. Banting immediately decided to share his half of the award with Charles Herbert Best.

## Type 1 diabetes

Type 1 diabetes is a chronic endocrine autoimmune disorder<sup>8, 9</sup> characterized by progressive loss of the pancreatic islet beta cells, resulting in insufficient insulin production to maintain normal blood glucose levels. According to the American Diabetes Association committee the term type 1A diabetes is recommended for immune mediated diabetes with its destruction of the islet beta cells of the pancreas. Non-immune mediated diabetes with severe insulin deficiency is termed type 1B<sup>10</sup>. In this thesis the term type 1 diabetes will be used and refers to immune mediated type 1A diabetes. The prevalence of type 1 disease in Sweden show that approximately 30,000 – 40,000 are afflicted<sup>11</sup>. The disease requires treatment with insulin, most often four–five injections per day to sustain life. Moreover, balancing the correct insulin dose with daily living activities in order to maintain a healthy blood glucose level involves close calculations and constant monitoring. Maintaining the blood sugar level close to normal dramatically reduces the risk of long-term complications which are either microvascular (e.g. retinopathy, nephropathy, and neuropathy) or macrovascular (e.g. cardiovascular disease, cerebrovascular accidents, and peripheral vascular disease). Thanks to improved treatment standards, these complications have become fewer and delayed over the past 20 years (reviewed in<sup>12</sup>). The disease is considered to be autoimmune since autoreactive T cells of inflammatory Th1 phenotype and islet autoantibodies to islet antigens have been found in individuals before they develop the disease as well as in newly diagnosed diabetic individuals<sup>13, 14</sup>. In addition, data from partial pancreas transplants between monozygotic twins showed that the non-diabetic pancreas was rapidly destroyed following transplantation and was accompanied by infiltration of the islets in the affected diabetic twin who received the transplant, called insulinitis, which is indicative of a strong autoreactive response<sup>15</sup>. Insulinitis is an inflammatory infiltrate of mononuclear cells<sup>16</sup> which leads to the selective destruction of insulin-producing beta cells in the pancreas and is regarded as the hallmark of recent type 1 diabetes onset<sup>16</sup>. In general, type 1 diabetes is considered a T cell mediated disease<sup>17</sup>. However, B lymphocytes play a crucial role as antigen-presenting cells as well. In fact, a recent study showed that selective depletion of B lymphocytes with rituximab (anti-CD20 monoclonal antibody Rituximab<sup>18</sup>) resulted in preserved beta-cell function in recent onset type 1 diabetes patients<sup>19</sup>. Examinations of insulinitis confirmed pancreatic tissue showed that the mononuclear cells were composed of CD4 and CD8 T lymphocytes, B lymphocytes, and macrophages<sup>20</sup>. Clinical onset of type 1 diabetes occurs at a threshold of decreased beta cell mass and early anatomy studies of the pancreas from patients implied that only ~10% of normal

beta cell mass remains shortly after clinical onset <sup>16</sup>. However, analyses have indicated that there may be continuous beta-cell function years after onset of type 1 diabetes, particularly in older individuals <sup>21,22</sup>. A useful estimate of insulin secretion is to utilize C-peptide <sup>2</sup> to describe changes in beta-cell function in type 1 diabetes patients <sup>23</sup>.

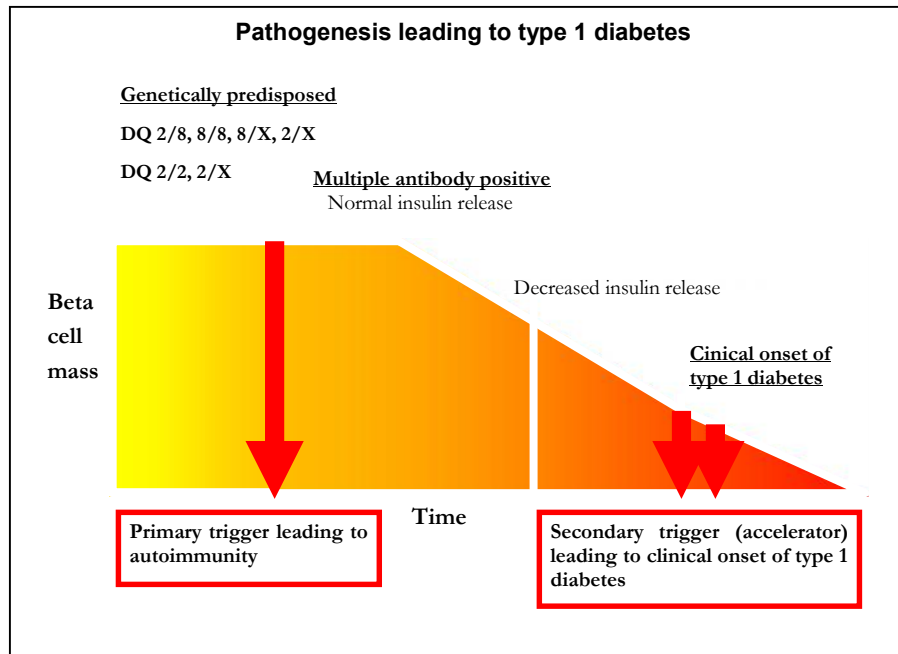


Figure 1. Schematic representation (adopted from Eisenbarth) of the pathogenesis leading to type 1 diabetes. Genetically predisposed individuals may first be exposed to a primary trigger leading to islet autoimmunity. The trigger may induce one or several islet autoantibodies. Multiple persistent islet autoantibody positivity may eventually be associated with a decreased insulin release. A second environmental trigger may accelerate the progression to clinical diabetes.

<sup>2</sup> C-peptide is the connecting polypeptide which is part of the proinsulin molecule. It is released when the A- and B-chains of insulin are cleaved off and facilitates the assembly, folding, and processing of insulin in the endoplasmic reticulum (ER).

## Incidence

According to a number of studies the incidence of type 1 diabetes is increasing worldwide<sup>24, 25</sup>. Several studies show a rapid increase amongst the younger children as well<sup>24, 26-28</sup>. The highest incidence is to be found in Finland<sup>29, 30</sup>. Sweden has the second highest incidence rate of childhood type 1 diabetes with an estimated incidence 40 per 100,000 in children less than 15 years of age<sup>11, 24</sup>. However, recent studies of all ages currently demonstrate that the disease is not becoming more frequent, but its median age at diagnosis has decreased. According to these studies, the increase in Sweden seems to have reached a plateau, concurrently with new onset patients have shifted to younger ages<sup>27, 31</sup>. There is a small male predominance, in newly diagnosed children, below 15 years of age. However, in those newly diagnosed after puberty there is a clear male excess with a ratio of 2 to 3:1<sup>32, 33</sup>. The explanation for this difference in sex ratio after puberty is not clear. It was also shown that there was a male majority with signs of humoral beta-cell autoimmunity among first-degree relatives older than ten years of age<sup>34</sup>.

Incidence rates vary between geographical regions and countries. A child in Finland has 40 times higher risk to develop type 1 diabetes than a child in Japan and almost 100 times more likely to develop the disease than a child in the Zunyi region of China<sup>25</sup>. Moreover, there was a close to six-fold gradient in the incidence of type 1 diabetes between Russian Karelia and bordering Finland, although the predisposing HLA-DQ genotypes are equally frequent in the two populations<sup>35</sup>. It was concluded that the rate of incidence was too rapid for genetics to explain the increase in disease incidence rate, consistent with the general view that environmental factors contribute to type 1 diabetes<sup>36-38</sup>.

## Genetics

The genetic etiology of type 1 diabetes is strongly associated with human leukocyte antigen (HLA) class II within the major histocompatibility complex (MHC), located on the short arm of chromosome 6<sup>39, 40</sup>. It includes approximately 3,500 kb of DNA and contains at least 150 genes. Many of the HLA genes are highly polymorphic and not only are they very closely linked, but their alleles are also in strong linkage disequilibrium, which complicates the identification of a possible primary disease-predisposing gene. Genome wide association studies have made it possible to identify additional genes on a variety of chromosomes but each one of these genes have lesser effects than HLA<sup>41</sup>. Of these, strong candidates after HLA are the insulin (INS) locus in 11p15<sup>42, 43</sup> and the CTLA4 gene in 6q27<sup>44, 45</sup>.

The first association between type 1 diabetes and HLA was reported for HLA-B8 and HLA-Bw15<sup>46, 47</sup> and negative associated with HLA-B7<sup>48</sup>. These were later recognized as part of the HLA class I region. Further studies revealed a stronger association with the HLA class II region. The genetic predisposition for type 1 diabetes was found to be particularly strong with the HLA-DQ A1\*0301-B1\*0302 (DQ8) and DQ A1\*0501-



B1\*0201 (DQ2) haplotypes<sup>41</sup>. The two haplotypes are found in nearly 90% of newly diagnosed type 1 diabetes children<sup>49, 50</sup>. Fewer than 40% of Caucasian background populations have these haplotypes<sup>8</sup>. The highest risk is conferred by the DQ2/8 genotype followed by homozygosity for both DQ8 and DQ2, as well as some but not all combinations of other haplotypes with either DQ8 or DQ2<sup>41, 51</sup>. The risk for type 1 diabetes is reduced by the presence of the DQ A1\*0102-B1\*0602 (DQ6.2) haplotype also with both DQ8 and DQ2<sup>52</sup>. In Caucasians DR4 showed the strongest risk association with type 1 diabetes, however, the DR4 haplotype represents more than 10 different DR4 subtypes (DRB1\*0401, 0402, 0403, 0404 etc). Anyone of these DR4 subtypes may be in linkage disequilibrium with e.g. DQ7, DQ8 or DQ9. For example, any DR4 subtype may occur with either DQB1\*0301 (DQ7) or with DQB1\*0302 (DQ8) but susceptibility is only related to the latter allele<sup>40, 53, 54</sup>. The risk allele DQB1\*0201 appears in both DR3-DQAI\*0501-DQB1\*0201 and DR7-DQAI\*0201-DQB1\*0201 but only DR3-DQAI\*0501-DQB1\*0201 is associated with type 1 diabetes risk therefore it was concluded that DQB1\*0201 is the susceptibility allele and not DQAI\*0501<sup>55</sup>. The effect of the two haplotypes DR4-DQB1\*0302 and DR3-DQB1\*0201 combined (DQ2/8 heterozygous) is greater than the sum of their separate effects<sup>56</sup>.

According to a number of studies associations between HLA and virus responsiveness has been reported. It has been shown that 96% of type 1 diabetes patients, positive for Coxsackie B virus-specific IgM at onset, had the HLA-DR3, DR4, or both haplotypes<sup>57</sup>. A recent study<sup>58</sup> suggests that diabetes-associated HLA-DR alleles were associated with a strong immune responsiveness and protective alleles with a weak responsiveness against EV antigens. Another study demonstrated an increased frequency of the ability of T lymphocytes to respond to mumps and Coxsackie B4 (CBV 4) when presented together with DR4, as compared with other DR determinants. In contrast, a decreased frequency was found in T lymphocytes responding to mumps or CBV 4 together with DR3, compared with other DR determinants<sup>59</sup>. However, the exact mechanisms by which HLA genes modulate diabetes risk are not known.

#### Islet autoimmunity

Islet autoantibodies are commonly found in pre-diabetic individuals<sup>60</sup>. These islet autoantibodies remain while beta cells are still distinguished but retract when the beta cells are gone<sup>61</sup>. At clinical onset of type 1 diabetes these autoantibodies are identified in approximately 98% of children<sup>50</sup> and 85% in adolescent<sup>62</sup>. Islet autoantibodies such as islet cell (ICA), glutamic acid decarboxylase (GADA), insulinoma antigen-2 (IA-2A), insulin (IAA) and zinc transporter 8 (ZnT8)<sup>63</sup> serve as warning signals for the risk of clinical type 1 diabetes<sup>64, 65, 66</sup>. Moreover the detection of islet autoantibodies in family members of affected type 1 diabetes patients, also identifies individuals with an elevated risk for type 1 diabetes<sup>8</sup>. GADA, IA-2A and IAA were analyzed in this thesis and are therefore described in more detail below. The numbers of islet autoantibodies are a part of the inclusion criteria currently used to classify type 1 diabetes<sup>67</sup> as they reflect the autoimmune pathogenesis of the disease<sup>68, 69</sup>. However,

the pathogenic involvement of the islet autoantibodies in beta-cell destruction is still controversial and the role of islet autoantibodies in the actual pathogenesis of type 1 diabetes has not been established in humans. Still, incomplete and sometimes complete beta-cell failure has been shown in islet autoantibody positive patients, whereas a lack of GADA or IA-2A at low titers indicated preservation of beta-cell function<sup>70</sup>. One study concluded that islet autoantibodies are not required for either the initiation or the progression of type 1 diabetes, by the observation that a patient with X-linked agammaglobulinemia<sup>3</sup> still developed type 1 diabetes in the absence of both islet autoantibodies and beta cells<sup>71</sup>. This conclusion was further supported by the findings that islet autoantibodies were not affected by cyclosporine therapy and that their presence in patients with type 1 diabetes of recent onset who were treated with cyclosporine or placebo for 12 months was not related to the subsequent remission of insulin-requiring diabetes or to the loss of glucagon-stimulated C-peptide response<sup>72</sup>.

## GADA

Glutamic acid decarboxylase 65 (GAD65) synthesizes gamma-aminobutyric acid (GABA) from glutamate in one single enzymatic step<sup>73</sup>. GABA mediates synaptic inhibitory and excitatory neurotransmission in the central nervous system via small vesicles at the synapses<sup>74</sup> and is also present in high concentrations in the pancreatic islet beta cells<sup>75</sup>. The discovery of GAD was through immunoprecipitation analysis of the 64 kilo Dalton (kDa) GAD protein in type 1 diabetes patients<sup>76</sup>. At least two isoforms of GAD exist in mammals, with molecular weights of 65 kDa (GAD65) and 67 kDa (GAD67). The genes coding for GAD65 and GAD67 show 65% identical amino acid sequences and suggests derive from a common ancestral GAD gene<sup>77, 78</sup>. Yet, GAD65 and GAD67 are encoded by two distinct genes located on human chromosomes 10 and 2, respectively<sup>77, 79, 80</sup> and show apparent discrepancy in expression as well. For instance, GAD65, but not GAD67 is a shared autoantigen in different autoimmune diseases. In Stiff-Person syndrome (previously termed Stiff-Man syndrome) GAD65 autoantibody (GADA) blocks the enzymatic activity of GAD65. However, in type 1 diabetes GADA doesn't seem to obstruct the enzyme activity<sup>81</sup>. Furthermore, GADA seems to react towards two different epitopes in the two autoimmune diseases<sup>73, 82</sup>. In type 1 diabetes the GADA epitope is conformational and localized in the C-terminal, while it appears to be linear in Stiff-Person syndrome and confined to the enzymatic active site<sup>83, 84</sup>. A further dissimilarity in two different autoimmune diseases is the titers of GADA. Patients with Stiff-Person syndrome have 100- to 500-fold higher titers of GADA than type 1 diabetes patients<sup>85</sup>. GAD65 and GAD67 also occur in the alpha and the delta cells, however GAD65 is in low level in these cells<sup>86</sup>. GAD65 is

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<sup>3</sup> X-linked agammaglobulinemia is a genetic disorder in which patients do not generate mature beta cells which manifests as a complete lack of antibodies.

found to be membrane-bound while GAD67 is soluble within the cytoplasm. GAD67 is also expressed in islet beta cells however in much reduced levels<sup>81</sup>. Features of GADA development in type 1 diabetes show associations with HLA-DR3 and HLA-DQ2<sup>87</sup> (risk haplotypes in type 1 diabetes)<sup>88</sup>. It also has relatively poor diagnostic sensitivity for type 1 diabetes in boys younger than ten years of age. GADA in girls have a high diagnostic sensitivity irrespectively of age at onset. This age dependent difference in sensitivity between boys and girls disappear in adolescence<sup>89</sup>. The highest sensitivity in GADA diagnostics has been observed in patients between 20 and 40 years of age<sup>90</sup>. Moreover it has been suggested that the positive predictive value of GADA in patients older than 45 years of age may be as high as 50%<sup>91</sup>. This is in agreement with the finding of an association between GADA and slower rate of disease progression<sup>92, 93</sup>. The reasons for this slower rate and higher frequency of GADA in older patients are at the moment elusive. It has been shown that the beta-cell surface lacks GAD65 expression, in so doing it makes it self inaccessible for the immune system under physiological conditions<sup>94</sup>. However, it is likely that GAD65 peptides are presented on the beta cell HLA Class I proteins. In this way GAD65 is accessible to T-cell Receptors (TCR) expressed on the surface of cytotoxic CD8<sup>+</sup> T cells. Lysis of the islet beta cells might release large amounts of GAD65, which could eventually lead to an autoimmune response against itself. This may explain the reason why GADA frequently appear delayed<sup>95</sup>. Today GAD65 is considered a major autoantigen in the disease<sup>73, 96</sup>.

#### IA-2A

Following further studies of the 64kDa GAD protein by trypsin treatment uncovered additional antigens in type 1 diabetes sera including a 40 kDa protein identified as a transmembrane protein insulinoma antigen-2 (IA-2) previously termed ICA512<sup>97-99</sup>. The IA-2 gene is situated on chromosome 2 and the protein consists of 979 amino acids with a molecular weight of 106 kDa. IA-2 is a ubiquitous intrinsic membrane protein of secretory granules neuroendocrine cells including pancreatic islets<sup>100</sup>. In type 1 diabetes patients the antigenic targets of IA-2 are mainly located within the protein tyrosine phosphatase (PTP) domain of the molecule, which resides in the cytoplasm<sup>101</sup>. Studies have tried to define the characteristic of IA-2A development in type 1 diabetes and reports show that IA-2A is associated with rapid progression to type 1 diabetes<sup>93</sup> as well as HLA-DR4<sup>88</sup> and HLA-DQ8<sup>102</sup>, that they are detected in a high frequency at diagnosis in type 1 diabetic children<sup>103</sup>, whereas the frequency is lower in adult-onset type 1 diabetic patients<sup>104</sup>. A multicenter study, Diabetes Antibody Standardization Program (DASP), assessed an evaluation of the new World Health Organization (WHO) reference reagent for autoantibodies to GAD and IA-2. Forty-six laboratories in 13 countries concluded that the workshop sensitivity of IA-2A in type 1 diabetes study was 58%, less than GADA but higher than IAA<sup>105</sup>. It is important to note that the sera tested in DASP are not representative of type 1 diabetes as they only represent patients willing to donate large volumes of blood. Young children are not represented in DASP. Other studies have shown that the diagnostic sensitivity of IA-2A was 64.4%<sup>106</sup>.

## IAA

Insulin autoantibodies (IAA) are defined as antibody against endogenous insulin, present in individuals not treated yet with exogenous insulin. In 1983, Palmer et al. were the first to publish low prevalence of IAA in the serum of type 1 diabetes patients, before receiving any insulin treatment<sup>107</sup>. The insulin gene is located on chromosome 11<sup>43</sup> and the protein consists of 51 amino acids with a molecular weight of 6 kDa. Insulin is the only known beta-cell specific autoantigen<sup>107</sup>. The characteristics for IAA association with development of type 1 diabetes are as follows: higher levels of IAA in patients are more likely to stay positive than those who are transiently positive<sup>108</sup>, correlates inversely with age and are significantly less prevalent in adult patients with newly diagnosed type 1 diabetes,<sup>107, 109, 110</sup> is dependent on gender<sup>111</sup> and higher levels of IAA is associated with DR4<sup>112</sup> and DQ8 alleles<sup>113</sup>. A prevalence of 81% in type 1 diabetes patients below ten years of age as compared to 61% in older patients was recently reported<sup>111</sup>. Furthermore, the incidence of IAA is comparable in both genders below 15 years of age but as the age increases the ratio of males to females that possess IAA is about 2:1<sup>111</sup>. The inverted correlation of IAA with age could be explained by the fact that IAA<sup>114-116</sup> is usually the first to appear, preceding the development of other islet autoantibodies, while later, they may decline<sup>114</sup>. The biological meaning of this dependence remains to be clarified.

Studying the development of islet autoantibody patterns shows that the number of antibodies, rather than the individual antibody, is more predictive in development of type 1 diabetes in which the risk is increased for every islet autoantibody obtained<sup>117, 118</sup>. They usually appear sequentially, rather than concurrently<sup>119</sup>. These observations has not been fully explained but one could definitely conclude that the islet autoantibodies GADA, IA-2A and IAA, are found in most patients with type 1 diabetes and are now established markers of preclinical type 1 diabetes<sup>50, 60, 62, 64-66, 107, 120</sup>.

### Islet autoantibodies in cord blood

Islet autoantibodies have proven to be transferred through the placenta from islet antibody positive mothers to their offspring<sup>121</sup>. It is furthermore generally believed that cord blood antibodies are of maternal origin since the fetus has a only a minor production of IgG and the active transport of IgG over the placenta barrier provides the fetus an IgG concentration of 150% of the mother's<sup>122-124</sup>. To determine whether exposure to islet autoantibodies modify the risk of type 1 diabetes in the offspring a number of studies have measured islet autoantibodies in cord blood. In a retrospective study it was shown that GADA and ICA in non-diabetic pregnancies predicted type 1 diabetes in the mother but not necessarily in the offspring<sup>125</sup>. A total of 42 type 1 diabetes mothers and their offspring were followed as well as 123 non-diabetic pregnant mothers as controls. The same investigators performed a similar study two years later but analyzed cord blood from islet autoantibody positive non-diabetic mothers instead<sup>126</sup>. Exclusion of mothers with type 1 diabetes was performed since this group of mothers often has islet autoantibodies before, during and after their

pregnancies. Cord blood from 81 children who developed type 1 diabetes before 15 years of age were analyzed and 17% had at least one islet autoantibody when born. It was concluded that children who develop type 1 diabetes have an increased prevalence of cord blood islet autoantibodies compared with control subjects. In contrast to these findings a study performed on 30 patients who developed type 1 diabetes between 15 and 30 years of age concluded that there was no evidence of an increased prevalence of islet autoantibodies in these young adults' cord blood<sup>127</sup>. The same conclusion was proposed by Diabetes Autoimmunity Study in the Young (DAISY) who suggested that the presence of cord blood autoantibodies did not predict the development of islet autoimmunity in children below five years of age. They also concluded that the majority of cord blood islet autoantibodies appeared to result from maternal transmission<sup>123</sup>. In a study of children born to mothers with type 1 diabetes it was demonstrated that offspring (n=678) who were GADA or IA-2A positive at birth had significantly lower risks for developing multiple islet autoantibodies and diabetes than offspring who were islet autoantibody negative at birth<sup>128</sup>. Their findings suggested that fetal exposure to islet autoantibodies in children born to mothers with type 1 diabetes may be protective against future islet autoimmunity and diabetes<sup>128</sup>. A large, population-based case-control cohort (2,023 cases and 4,042 controls) in which all type 1 diabetes children and adolescents born in Denmark between 1981 and 2002 were identified concluded the opposite. The study showed that GADA and IA-2A positivity at birth was associated with a 7.5-fold increased risk for developing type 1 diabetes<sup>129</sup>.

#### Infection as a possible trigger of type 1 diabetes

Even though genetic predisposition is a major risk factor of type 1 diabetes, the pair wise concordance between monozygotic twins show that only 30–50% of monozygotic twins are at risk for the disease<sup>37, 130</sup>, indicating that environmental factors might be involved<sup>36-38</sup>. Additional observations such as a seasonal variation in the diagnosis of the disease<sup>131</sup>, a 10-fold difference in the occurrence of type 1 diabetes in various parts of Europe<sup>37</sup> and neighboring countries with quite similar HLA genotypes but noticeable difference in the incidence of diabetes<sup>132</sup> further contribute to the environmental risk factor. Several viruses have been associated with type 1 diabetes. Listed in Table 1 are viruses other than EV implicated to be associated, or not confirmed associated, with type 1 diabetes. Some viruses are diabetogenic in animals, and others have been implicated as triggers in humans. Two viruses implicated to cause type 1 diabetes are rubella and mumps. Gundersen reported in his study from as early as 1927 an increase in the number of cases with type 1 diabetes two to four years after a mumps epidemic (cited in<sup>37</sup>). Other early observations were the associations between congenital rubella infections and a high rate of subsequent type 1 diabetes<sup>133, 134</sup>. Approximately 12–20% of individuals infected with congenital rubella will subsequently develop type 1 diabetes within a range of five to 20 years<sup>135, 136</sup>. In an attempt to understand the potential role of rubella virus in type 1 diabetes one study examined the cross-reaction between viral and beta-cell protein determinants and concluded that cross-reactive GAD65 and rubella virus determinants identified by

T cell clones were also recognized at high frequencies by general T cell populations of type 1 diabetic patients<sup>137</sup> while an additional study showed that mumps was involved in cytokine release from human beta cells<sup>138</sup>. The incidence of congenital rubella and mumps has fallen dramatically due to the measles–mumps–rubella (MMR) vaccination program. Although congenital rubella infection leads to rate of diabetes of up to 20%, its successful vaccination did not decrease the incidence of type 1 diabetes. Furthermore, there is no suggestion that the MMR vaccination programs have resulted in type 1 diabetes incidence increase<sup>139, 140</sup>. Recent epidemiological study showed that rotavirus infections, the most common cause of childhood gastroenteritis, appeared to be highly significant associated with seroconversion and increases in islet autoantibodies IA-2A, GADA and IAA in genetically susceptible children<sup>141</sup>. However this correlation was not observed in another report, in which type 1 diabetes genetically susceptible children were studied<sup>142</sup>. To determine if infections were associated with the risk of type 1 diabetes the childhood the EURODIAB substudy 2 studied variables collected either by interview or questionnaire. They concluded that infections early in the child's life were associated with an increased risk of type 1 diabetes. Additional contrasting findings showed that pre-school day-care attendance, a proxy measure for total infectious disease exposure in early childhood, was found to be inversely associated with risk of developing type 1 diabetes<sup>143</sup>. An additional report, also supervised by questionnaire handling, conducted by the DAISY study concluded that maternal symptoms of common infections during pregnancy showed a lower risk of developing islet autoimmunity in the young girls<sup>144</sup>.

Table 1. Other viruses than enterovirus reported to be associated/not associated with type 1 diabetes

Virus	Association	No association
Mumps virus	Helmke et al. 1980 <sup>145</sup> Hyöty et al. 1988 <sup>146</sup>	Hiltunen et al. 1999 <sup>140</sup>
Rubella virus	Menser et al. 1974 <sup>133</sup> , 1978 <sup>135</sup> Ginsberg-Fellner et al. 1984 <sup>134</sup> McIntosh et al. 1992 <sup>136</sup> Ou et al. 2000 <sup>137</sup>	Hiltunen et al. 1999 <sup>140</sup> Viskary et al. 2003 <sup>147</sup>
Rota virus	Honeyman et al. 2000 <sup>141</sup> Coulson et al. 2002 <sup>148</sup>	Blomqvist et al. 2002 <sup>142</sup>
Cytomegalo virus	Ward et al. 1979 <sup>149</sup>	Ivarsson et al. 1993 <sup>150</sup>
Retrovirus	Hao et al. 1993 <sup>151</sup> Conrad et al. 1997 <sup>152</sup>	Badenhoop et al. 1999 <sup>153</sup>
Parvovirus B19	Onitjol et al. 2005 <sup>154</sup> Munakat et al. 2005 <sup>155</sup>	O'Brayan et al 2005 <sup>156</sup>
Epstein-Barr virus	Surcel et al. 1988 <sup>157</sup> Horn et al. 1988 <sup>158</sup>	Sairenji et al. 1991 <sup>159</sup>

#### Enteroviruses

The human enterovirus genus is a member of the Picornaviridae family of small, icosahedral, single-stranded, positive-sense RNA viruses. The morphology of the virions in this family is described as a non-enveloped capsid with an icosahedral symmetry. In an icosahedral EV capsid (with a diameter of 28-30 nm), there are 12 pentamers, each pentamer is composed of five protomers and each protomer consists of one copy out of the four capsid proteins (VP1, VP2, VP3, VP4 (VP4 is located on the internal side of the capsid)). According to the International Committee on Taxonomy of viruses (<http://www.ictvdb.org/ictv/index.htm>), other genus members of the Picornaviridae are Cardiovirus, Aphovirus, Hepatovirus, Parechovirus, Erbovirus, Kobuvirus and Techovirus. EVs are classified as five different species (poliovirus, EV-A, -B, -C and EV-D) on the basis of phylogenetic properties. More recently two human rhinovirus species were merged with EV A-D in the genus of EVs. Identifying new EV serotypes was previously done by antibody neutralization test <sup>160</sup> which was labor-intensive and time-consuming. Due to new techniques and the development of molecular typing systems the time required to type a new EV isolate has been reduced. Up until 2008, approximately 100 different EV serotypes was identified <sup>161</sup>. The seasonal incidence of EV infections peaks in the late summer and autumn <sup>162</sup>. EV

infections are more frequent in childhood and can be acquired as early as neonatally or even in utero <sup>163, 164</sup>. In adults most EV infections are asymptomatic or produce subclinical, mild symptoms, although the infections are also known to provoke acute diseases such as meningitis, encephalitis and pericarditis <sup>165</sup>. EV is thought to play an important part in the pathogenesis in type 1 diabetes, CBV 4 being the most frequently implicated serotype <sup>166</sup>. The first reports of virus induced diabetes in animals was according to Gamble <sup>167</sup> published already in 1962 by Barboni and Manoccio where cattle developed diabetes following foot and mouth disease which is a picornavirus serotype (cited in <sup>167</sup>). Another study published four years later, showed that another picornavirus, encephalomyocarditis virus, caused diabetes mellitus by reducing the mass of functional beta cells of the islets of Langerhans in mice <sup>168</sup>. Studies until now have suggested associations not only between EV infections and manifestation of clinical type 1 diabetes <sup>169-171</sup>, but also between EV infections and the initiation of the autoimmune process where EV infections appear to coincide with seroconversion to islet autoantibodies <sup>172, 173</sup>. In addition, studies have reported associations between gestational EV infection and type 1 diabetes in the child have been reported as well <sup>174, 175</sup>. The associations between EV infections and triggering of type 1 diabetes will be described in more detail below and the focus will be on the three possible time points in which EV infections may lead to the development of either islet autoimmunity or clinical onset of type 1 diabetes. The three possible time points would be: gestational EV infections and type 1 diabetes in the offspring, EV infections trigger of islet autoimmunity and EV infections as an accelerator to clinical type 1 diabetes (Table 2a – 2c).

Enterovirus – a possible association between gestational infections and type 1 diabetes in the offspring

Despite reports of intrauterine transmission of EVs in animals <sup>176</sup>, transplacental infection in humans is not well accepted. However, number of case reports have concluded following: detection of EV in stillbirth infants at autopsy and in the placental tissues of newborns with neuro-developmental delays <sup>177</sup>, CBV 3 has been isolated from a stillborn infant with myocarditis and hydrops fetalis <sup>178</sup>, post-natal diagnosis of congenital skin lesions caused by intrauterine CBV 3 infection <sup>179</sup>, CBV 3 detected in a stillborn hydropic fetus <sup>180</sup> and a recent case of intrauterine CBV 3 infection diagnosed during the second trimester with nonimmune fetal hydrops, resulting in a live birth and subsequent early neonatal death <sup>181</sup>, all supporting intrauterine transmission of EV. Few studies have investigated gestational EV infections leading to development of type 1 diabetes in the child. The first report used the nationwide Swedish Childhood diabetes register and published in April 1995 (Table 2a) <sup>182</sup>. Maternal serum from 57 mothers at delivery of children who developed type 1 diabetes before 15 years of age were compared with serum from 203 mothers of control subjects who were delivered at the same hospital during the same time period. The group-specific enzyme-linked immunosorbent assay for enteroviral IgG and IgM antibodies in this study showed that EV IgM and IgG antibodies against enteroviral antigens (echovirus 9 and 30, CBV 5) were significantly higher among mothers whose



children later developed type 1 diabetes. The second report was conducted by the prospective population-based Childhood Diabetes in Finland study (DiMe) in June the same year <sup>174</sup> which showed an association between EV infections in the mother and type 1 diabetes in the child when presented with disease before three years of age, but not in group of four to six years of age. The serum samples had been collected from mothers at the end of the third month of pregnancy to 90 type 1 diabetes children. The control mothers were matched for time of delivery ( $\pm$  one day) and gender of offspring. Samples were analyzed for IgG, IgM, and IgA class antibodies by a capture-based RIA method. By the end of the same year the Swedish Childhood diabetes register published a similar paper <sup>175</sup> as their first one, with approximately the same number of mothers, matched to their control mothers no differently from the previous study but the IgM analysis this time covered CBV 2-4. They concluded a significantly higher frequency of CBV 3 IgM at delivery in mothers whose children later developed type 1 diabetes compared to their matched controls, but not of CBV 2 and CBV 4. An additional report on samples collected in early pregnancy was conducted four years later, once more by the Swedish Childhood diabetes register. A case-control study in which serum samples were collected during the first trimester from 85 mothers whose children developed type 1 diabetes before 15 years of age and compared to 172 controls of mothers whose children did not develop type 1 diabetes <sup>183</sup>. The samples were analyzed for CBV with RNA by reverse transcription-polymerase chain reaction (RT-PCR) and EV IgA, IgM and IgG by ELISA. They found that three mothers of type 1 diabetes children were CBV RNA positive and another three were positive for CBV IgM antibodies. Thus six out of 85 mothers of type 1 diabetes children had signs of EV infection in early pregnancy compared with one of 172 controls. Diabetes Incidence Study in Sweden (DISS) study recently published a report <sup>184</sup> in which Elfving et al analyzed serum samples collected at delivery from 30 non-diabetic mothers whose offspring developed type 1 diabetes between 15 and 25 years of age and compared them to 90 maternal serum samples matched by date of delivery. The samples were analyzed for EV-IgM and EV-RNA. Among the 30 non-diabetic mothers 30% were EV-IgM positive, and none was positive for EV-RNA. In the control group, 16% were EV-IgM positive, and 4% were positive for EV-RNA. None of these results were significant however boys of EV-IgM positive mothers had approximately five times greater risk of developing diabetes as compared to boys of IgM negative mothers. It should be noted that studies have concluded negative results in the association between gestational EV infections and type 1 diabetes in the offspring. The German multicenter BABYDIAB found no evidence for an association of EV infections during pregnancy and early childhood with development of islet autoantibodies in offspring <sup>185</sup>. The study estimated EV infections from birth, prior to and in parallel with the appearance of islet autoantibodies in offspring of parents with type 1 diabetes by IgG antibodies against a panel of CBVs, and IgG and IgM antibodies to CBV 3, CBV 4, and CBV 5. The aim of a Finnish study was to evaluate the role of first trimester EV infections in two series of pregnant women <sup>186</sup>. The first series of mothers were 948 women whose child developed clinical diabetes before the ages of 15 and the second series 680 women whose child developed clinical diabetes before seven years of age. IgM class antibodies against CBV 5 and a mixture of CBV 3,

CAV 16, and echovirus 11 antigens were analyzed. The results suggested that first trimester maternal EV infection was not a risk factor for type 1 diabetes in the offspring.

Table 2a. Positive associations between gestational enterovirus infections and type 1 diabetes in the offspring.

Pregnancy/Delivery	Virus/Method	Reference
Early pregnancy		
Swedish Childhood diabetes register	CBV by PCR and IgA, IgM, and IgG by ELISA	Dahlquist G et al. Diabetes Care 1999 <sup>183</sup>
Late pregnancy		
DiMe study	IgG, IgM and IgA capture radioimmunoassay	Hyoty H et al Diabetes 1995 <sup>174</sup>
Delivery		
Swedish Childhood Diabetes register	CBV 2, 3 and 4 by IgM capture radioimmunoassay	Dahlquist G et al Diabetologia 1995 <sup>175</sup>
Swedish Childhood Diabetes register	IgG and IgM towards echo9, echovirus 30, CBV 5	Dahlquist G. et al Diabetes 1995 <sup>182</sup>
DISS study	EV by PCR and IgM capture immunoassay	Elfving M et al. Exp Diabetes Res 2008 <sup>184</sup>

#### Enterovirus – as a possible trigger of islet autoimmunity

A further triggering time point has been observed between EV infections and the initiation of the autoimmune process where infections appear to coincide with seroconversion to islet autoantibodies <sup>172, 173, 187, 188</sup>. Association between EV infections and seroconversion of islet autoantibodies was reported as early as 1982. In this report a family was described in whom serial measurements of ICA and CBV 3, 4 and 5 titers were determined for three years before one of the children developed type 1 diabetes <sup>189</sup>. Few years later a fatal case of CBV 6 infection with characteristic features of a viral meningoencephalitis also demonstrated ICA seroconversion during hospitalization in a young child <sup>190</sup>. One already mentioned study is the DiMe study (Table 2b) <sup>174</sup> that not only showed an association between EV infections in the mother and type 1 diabetes in the offspring but also demonstrated concurrent infections associated with an ICA response in at least one-third of the pre-diabetic children, while they were rare in the control subjects. In this study controls were

matched for age, gender, and the time period during which samples were collected. The same study followed siblings of type 1 diabetes patients who seroconverted to ICA during a prospective follow-up<sup>173</sup>. The cohort consisted of 765 non-diabetic siblings and comprised blood sampling every six months. IgG, IgM, and IgA class antibodies were analyzed for a panel of EV antigens. Increase in EV antibody levels were significantly more frequent in sample intervals in which ICA first appeared than in sample intervals in ICA negative control sibling. The children who converted to ICA during an EV infection more often had the DQ2/8 genotype than children who stayed ICA negative. The Finnish Diabetes Prediction and Prevention Study (DIPP) investigated the role of EV infections in children who have tested positive for islet autoantibodies in a prospectively starting at birth<sup>172</sup>. Samples were drawn at birth and subsequently every three to six month interval. IgG and IgA class antibodies against purified CBV 4, purified echovirus 11 and an EV peptide antigen derived from an immunodominant region of capsid protein VP1<sup>4</sup> were measured by enzyme immunoassay (EIA). Presence of EV-RNA was analyzed as well by RT-PCR. The cohort consisted of serum from 21 children who developed and retained islet autoantibodies and 104 control subjects matched for the time of birth, gender, and HLA. An association between EV infections and the induction of autoimmunity was found as EV infections were detected in 57% of the case subjects during a six month follow-up period preceding the first appearance of islet autoantibodies compared with 31% of the matched control children. The conclusion demonstrated that EV infections were associated with the development of beta-cell autoimmunity and would provide evidence for the role of EVs in the initiation of beta-cell destruction. The international study Trial to Reduce IDDM in the Genetically at Risk (TRIGR) evaluated in one report the risk effect of EV infections in children who were followed prospectively from birth and subsequently developed signs of progressive beta-cell autoimmunity, i.e. positivity for type 1 diabetes associated autoantibodies by the age of two years<sup>191</sup>. The 103 children included 19 cases and 84 control subjects. Their serum samples were analyzed for IgG and IgA towards CBV 4 and echovirus 11 as well as for EV-RNA. The result showed that autoantibody positive children had more EV infections than autoantibody negative children before the appearance of autoantibodies which suggested an association between EV infections and induction of beta-cell autoimmunity. Three years after the DIPP study reported that EV infections were associated with the development of beta-cell autoimmunity<sup>172</sup>. They conducted a similar approach as previously but with the double amount of cases as well as controls<sup>187</sup>. Their conclusions were comparable with their earlier effort with the exception of a stronger significance in association. The first study to evaluate the role of viral infections as accelerating an already initiated disease process in humans and to follow the rate of progression from islet autoimmunity to clinical diabetes was performed by DAISY and published in December 2010<sup>171</sup>. The cohort comprised children who tested positive for one or more islet autoantibodies on two or more consecutive clinic

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<sup>4</sup> VP1 is a common epitope for several EVs

visits. Blood samples and rectal swabs were collected every three to six months after seroconversion for GADA, IAA or IA-2A until diagnosis of diabetes. The rate of progression from islet autoimmunity to diabetes was found to be significantly increased after the detection of EV-RNA in serum but not after detection of EV-RNA in rectal swab samples. The observation led to the conclusion that progression from islet autoimmunity to type 1 diabetes may increase after an EV infection characterized by the presence of viral RNA in the serum. However, conflicting results in observations between EV infections and the initiation of the autoimmune process have also been obtained. The DAISY study also investigated whether there was an association between EV infections and beta-cell autoimmunity in children at higher risk of developing type 1 diabetes <sup>192</sup>. A nested matched case-control study of incident cases of beta-cell autoimmunity within two prospective cohorts of genetically high-risk children (cases=26, controls=39). EV infection was detected by PCR of serum, saliva and rectal swab samples. The study showed no evidence that EV infection would be a risk factor for the development of beta-cell autoimmunity. The Norwegian MIDIA study tested whether the frequency of EV-RNA was associated with development of multiple islet autoantibodies in children with the highest HLA risk genotype in fecal samples from early infancy <sup>193</sup>. The study included 27 children who developed two or more islet autoantibodies in two or more consecutive samples and two control subjects per case matched by follow-up time, date of birth, and county of residence. The conclusion showed no support for higher frequency of EV in children who developed two or more islet autoantibodies than in control subjects. An additional MIDIA study recently published a nested case-control study, which included 27 children who developed islet autoimmunity and 53 children matched for age and community of residence <sup>194</sup>. The objective of this study was to investigate a possible association between human parechovirus infections in early infancy and the development of islet autoimmunity. Subsequently analyzing monthly stool samples for human parechovirus using a semi-quantitative RT-PCR they concluded that there was no associations between human parechovirus infections and the signs of islet autoimmunity. It is noted that parechovirus is another genus member of the Picornaviridae family.

Table 2b. Enterovirus as a possible trigger of islet autoimmunity.

Development of islet autoimmunity		Virus/Method	Reference
DiMe study	IgG, IgM and IgA capture radioimmunoassay		Hyoty H et al Diabetes 1995 <sup>174</sup>
DiMe study	IgG, IgM, IgA towards CBV 1-5 CAV 9, 16. echovirus 9, 18 and 30		Hiltunen M et al. J Infect Dis 1997 <sup>173</sup>
Case report	EV IgM and IgG		Lönnrot M et al. Diabet Med 1998 <sup>195</sup>
DiMe study	EV RT-PCR		Lönnrot et al. J Med Virol 2000 <sup>188</sup>
DIPP study	EV RT-PCR		Lönnrot et al. Diabetes 2000 <sup>172</sup>
TRIGR project	IgG and IgA towards CBV 4 and echovirus 11 RT-PCR		Sadeharju et al. Clin Exp Immunol 2003 <sup>191</sup>
DIPP study	EV RT-PCR		Salminen et al. J Med Virol 2003 <sup>187</sup>
Case report	Echovirus 3 by neutralizing antibodies and RT-PCR		Williams CH et al. J Clin Microbiol 2006 <sup>196</sup>

### Enterovirus – as a possible accelerator of type 1 diabetes clinical onset

The possibility that an EV infection may be associated with the onset of type 1 diabetes in humans was first published in 1969 <sup>197</sup>. The study, which tested for neutralizing antibody to CBV 1-6, concluded that insulin-dependent diabetes within three months of onset was found to have higher antibody titers, particularly CBV 4, than controls <sup>197</sup>. The same serotype was isolated from the pancreas of ten year old boy who was admitted to the hospital in ketoacidosis in a study published in 1979 (Table 2c) <sup>198</sup>. Neutralization analyses showed increasing levels of CBV 4 during the month of hospitalization. Post-mortem examination showed lymphocytic infiltration of the islets of Langerhans and necrosis of beta cells. Mice inoculated with the human isolate showed hyperglycemia, inflammatory cells in the islets of Langerhans and beta-cell necrosis. Staining pancreatic sections of the mouse revealed viral antigens in beta cells. Later studies verified CBV 4 isolated from the pancreas of type 1 diabetes patients <sup>199</sup> and in postmortem pancreas samples from patients with type 1 diabetes by in situ hybridization but not in samples from individuals without the disease <sup>200</sup>. Following studies confirmed the association between EV infections and type 1 diabetes <sup>57, 201-205</sup>. The majority of these studies were usually evaluated as case-controls studies and the association was based largely on the detection of specific anti-CBV IgM or neutralizing antibodies in serum of children at the onset of type 1 diabetes <sup>202, 205</sup>. A greater part of the cases included in these studies were poorly matched to their controls and included matching variables such as, similar age group, gender and sampling date. Applying RT-PCR amplification of new EV genomic sequences made it possible to detect a broad array of EV serotypes <sup>206, 207</sup>. In 1995 Clements et al. reported that infection with CBV was linked to type 1 diabetes. Serum samples from children below six years of age were analyzed for EV by PCR. Nine of 14 serum samples (64%) taken from

children at the onset of type 1 diabetes were positive for EV-RNA compared to two of 45 control children (4%)<sup>208</sup>. The detection of EV-RNA in a later study was carried out in adult newly diagnosed type 1 diabetes patients using seminested RT-PCR<sup>209</sup>. A more refined case-control study included serum from 110 children obtained shortly after diagnosis and compared to 182 controls matched for age, geographical location and time of year<sup>210</sup>. The occurrence of EV-RNA was analyzed by 5' nontranslated region (5' NTR) PCR and the results showed that a significantly larger number of type 1 diabetes children (27% vs. 4.9%,  $p < 0.005$ ) had evidence of EV-RNA.

The majority of studies on EV associations with type 1 diabetes reviewed so far have been based on the detection of EV by EV specific antibodies or PCR, and some studies identified EV in the pancreas of diabetic subjects. A study by Oikarinen et al published in 2007 also detected EV in small-intestine biopsy samples from patients with type 1 diabetes compared to healthy individuals<sup>211</sup>. The biopsies were analyzed by in situ hybridization, immunohistochemistry and RT-PCR. The presence of EV was confirmed by all three methods and the results suggest a persistent EV infection of the digestive mucosa membrane of the patients. This conclusion wouldn't seem unlikely as EV is transmitted principally through the fecal-oral route and the primary replication site is in the gut mucosa. In addition, previous studies supports enhanced immune activation of the small intestine in type 1 diabetic patients<sup>212, 213</sup> which further could be explained by a local virus infection in intestinal mucosa.

The mechanisms of which EV infections would lead to mothers conferring type 1 diabetes risk to her fetus, islet autoimmunity or clinical onset of type 1 diabetes are still poorly understood. Some studies are too small and have serious limitations in study design, in particular in the selection of control subjects. For a number of the studies, matching controls were not adequate and had not considered confounding factors such as genetic predisposition in HLA, age and the date of sampling. This is very important since HLA presents pathogens to the immune system and the incidence of EV infection is known to vary with age, season and geographical area. Furthermore, in contrast to some of these studies other reports observed no associations between EV infections and manifestation of clinical type 1 diabetes. The DiMe study collected serum samples from 47 newly diagnosed type 1 diabetes children with the aim to determine if EV infections precede clinical type 1 diabetes. The samples were analyzed by RT-PCR and neutralizing antibodies against CBV serotypes 1–6. However, no association was found between EV infection and manifestation of clinical type 1 diabetes<sup>188</sup>.

Table 2c. Associations between EV infections and the clinical onset of type 1 diabetes as well as after clinical onset.

At clinical onset	Virus/Method	Reference
Case report	CBV 4 by neutralizing antibodies	Yoon J-W et al. N Engl J Med 1979 <sup>198</sup>
Case report	CBV 5 by neutralizing antibodies	Champsaur H et al. Lancet 1980 <sup>214</sup>
Case-control	CBV 1-6 IgM by ELISA	King ML et al Lancet 1983 <sup>203</sup>
Case-control	Capture-IgM RIA and neutralization tests CBV 4	Frisk G et al. J Infect Januari 1997 <sup>215</sup>
Case-control	IgM-capture CBV 1-5	Frisk G et al. Diabetologia March 1992 <sup>201</sup>
Pittsburgh Diabetes Research Group	CBV 1-6, CAV 9, echovirus 4, 6, 9, 11, 30, 34, and EV 71 IgM by ELISA	Helfand et al J Infect Dis 1995 <sup>205</sup>
Case-control	CBV 3 and B 4 by PCR	Clements GB et al. Lancet 1995 <sup>208</sup>
Case-control	CBV 3 and 4 by seminested RT-PCR	Andreoletti L et al. J Med Virol 1997 <sup>209</sup>
Case-control	CBV 2 by RT-PCR 5' NCR	Nairn C et al. Diabet Med 1999 <sup>210</sup>
Case-control	CBV 2-4 by seminested RT-PCR	Cehadeh J Infect Dis 2000 <sup>216</sup>
Case-control	RT-PCR 5' NCR	Yin H et al Diabetes 2002 <sup>169</sup>
Case report	Echovirus 30 by neutralizing antibodies	Cabrera-Rode et al. J Med Virol 2005 <sup>217</sup>
Case report	CBV 4 and 5 by RT-PCR neutralizing antibodies	Hindersson et al. J Clin Virol 2005 <sup>218</sup>
DAISY	RT-PCR 5' NCR	Stene LC et al Diabetes 2010 <sup>219</sup>
After clinical diagnosis		
	CBV 1-6 by neutralizing antibodies	Gamble DR, et al Br Med J 1969 <sup>197</sup>
	CBV 4 by Immunohistochemical, electron microscopy	Dotta F et al. Proc Natl Acad Sci 2007 <sup>197</sup>
Case-control	In situ hybridization, immunohistochemistry and RT-PCR	Oikarinen et al. Clin Exp Immunol 2007 <sup>211</sup>

## Experimental animals and type 1 diabetes

The two well known rodents commonly used to study mechanisms of type 1 diabetes are the non-obese diabetic mouse (NOD-mouse) (reviewed in <sup>220</sup>) and the BioBreeding rat (BB-rat) (reviewed in <sup>221</sup>). These animals have allowed comprehensive study of the communication between cells of both the innate and acquired immune system that cause and generates and release of pancreatic beta-cell reactive T cells. CBV infection (B4 strain in particular) of the NOD mouse has shown to induce diabetes <sup>222</sup>. However, treating younger NOD mice with the same virus prevents diabetes, implying that the timing of infection is essential in disease development <sup>223</sup>. Experimental animals are certainly useful to study mechanisms of disease processes. However, both mice and rats are poor models of human infectious diseases, as EV including poliovirus does not always infect rodents.

### Other specific agents or events associated with triggering the clinical onset of type 1 diabetes

Interest in how dietary factors could modify the diabetogenicity in rodents began as early as the 1940s <sup>224, 225</sup>. In humans, weight gain was one of the first concepts which showed that virus infections had nutritional effects <sup>226</sup>. The observation from the early 1970s presented an association between accelerated weight gain during infancy in children who later developed type 1 diabetes compared with control children <sup>226</sup>. This observation was later confirmed and expanded to comprise increased height during childhood as a risk predictor as well <sup>227-229</sup>. So far, these data needs to be verified in prospective studies such as The Environmental Determinants of Diabetes in the Young (TEDDY) study <sup>230</sup>. The reports listed in Table 3 include agents other than viruses considered important for triggering the clinical onset type 1 diabetes. Listed are studies which either concluded consistent or conflicting reports on the various agents and events considered important for triggering clinical onset type 1 diabetes other than viruses. Some of the larger studies which are taken into account in Table 3 include the nationwide Swedish Childhood Diabetes Registry, the EURODIAB Substudy 2, and the Bart's-Oxford (BOX) family study. The nationwide Swedish Childhood Diabetes Registry, include 99% of recent-onset type 1 diabetes patients (0-14 years) in Sweden and was linked with the Swedish Medical Birth Registry. The matched case-control study was conducted analyzing about 20 perinatal variables concerning mother and child and a total of 2,757 type 1 diabetes children were analyzed <sup>231</sup>. The EURODIAB Substudy 2 is a large multicenter case-control study including eight participating centers: Austria, Latvia, Lithuania, Luxembourg, Romania, Bulgaria, Northern Ireland, U.K. Hospital records concerning the mother's pregnancy and the child's perinatal history from 1,093 children with type 1 diabetes compared to 3,264 control subjects were studied <sup>228</sup>. The prospective population based BOX family study of childhood diabetes has since 1985 recruited more than 90% of the families of children who have developed type 1 diabetes under the age of 21 in the former Oxford



Regional Health Authority area. Data, including date of birth and history of diabetes and an annual questionnaire was analyzed for 3,221 offspring from 1,375 families<sup>232</sup>.

Table 3. Specific agents and events considered important for triggering clinical onset type 1 diabetes other than viruses.

	Consistent report	Conflicting report
<b>Perinatal factors and postnatal growth</b>		
Older maternal age at birth	228, 231, 233, 234	235
Type 1 diabetes in the mother	231, 236, 237	128, 238
Maternal weight gain	239	231, 235
Birth order (first born)	228	231, 232, 234, 240
Gestational length	240, 241	228, 242
Maternal-fetal blood group incompatibility	228, 231	
Preeclampsia	228, 231, 243	244
Cesarean section	231, 234	228, 244
Low birth weight (protective)	228	245, 246
<b>Dietary factors</b>		
Short breastfeeding and/or early cow's milk introduction increases type 1 diabetes risk	247, 248	249
Early introduction of foods containing gluten or cereal before the age of three months	249, 250	251
N-nitroso compounds	252, 253	254
Early vitamin D supplementation decreased type 1 diabetes risk	255, 256	257
<b>Psychosocial factors</b>		
Divorce and violence	258, 259	
Family illness or loss of a close relative	260	
Low paternal education level and foreign origin of the mother	261	

## Potential mechanisms in virus-induced autoimmunity

Virus infections have been long associated with autoimmune diseases such as multiple sclerosis (MS), myocarditis and type 1 diabetes. Summarized below are perspectives on three potential mechanisms for virus-induced autoimmune disease. These include molecular mimicry, bystander activation, and the hygiene hypothesis.

### Molecular mimicry

Molecular mimicry is characterized by shared immunologic epitope with a microbe and the host <sup>262</sup> and according to the model an initial immune response against microbe proteins sharing similarity with self-proteins, (either linear amino acid sequence or their conformational fit) would trigger autoreactive T and B cells. These autoreactive cells would then be able to destroy self-tissue even after clearance of the viral infection. As previously mentioned CBV 4 was isolated from the pancreas of ten year old boy with ketoacidosis and the isolates were able to induce diabetes in susceptible mouse strains<sup>198</sup>. An infection with the same serotype increases the expression of GAD65 in mice <sup>263</sup>. Furthermore studies verified CBV 4 isolated from the pancreas of type 1 diabetes patients <sup>199</sup> and in postmortem pancreas samples of type 1 diabetes patients <sup>200</sup> which suggest the possibility of the virus to home to islets. There is a significant sequence homology between the viral protein P2-C of CBV 4 virus and GAD65 <sup>264, 265</sup> (Figure 2).

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Human GAD65 residues 250-273  A M M I A R F K M F P E V K E K G M A A L P R L
                               . . . . . | . . | | | | | . | | |
CBV 2C residues 28-50       F I E W L K V K I L P E V K E K H E F - L S R L
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Figure 2. Sequence homology between the viral protein P2-C of CBV 4 virus and GAD65. Lines show identical amino acid residues, while dots indicate amino acid residues with similar charge, polarity, or hydrophobicity.

The possible role of amino acid sequence and epitope homologies was studied in mice and type 1 diabetes patients by use of peptide antibodies to the P2-C, GAD65, and GAD67 <sup>266</sup>. All three peptide antisera reacted very strongly with homologous peptides in humans; P2-C antiserum cross-reacted with GAD65 as efficiently as GAD65 antiserum with P2-C, but no cross-reaction was detected between P2-C and GAD67. The results suggested that molecular mimicry may play a role in the pathogenesis of the disease <sup>266</sup>. However, by using monoclonal antibodies to GAD65 from a type 1 diabetes patient it was later shown that even though the homologous GAD65 region to P2-C was the target of islet autoantibodies, the antibodies showed no cross-reactivity with native viral antigens of CBV 1-6 <sup>267</sup>. Furthermore, IgM-positive human sera against CBV 4 did not cross-react with recombinant GAD65 <sup>268</sup>.

## Bystander activation

Is a direct viral infection of the beta cells necessary for the development of type 1 diabetes? Infections of beta cells surrounding tissue might lead to release of immune mediators like interferon-gamma (IFN- $\gamma$ ), tumor necrosis factor-alpha (TNF- $\alpha$ ), and nitric oxide (NO), which have been shown to be harmful to in vitro cultured beta cells<sup>269, 270</sup>. If the infection elicits a strong cytokine response locally, it can lead to an increase and activation of pre-existing autoreactive T cells which can be found in all humans. These autoreactive T cells managed to evade the negative selection during their maturation in the thymus since their affinity to the self determinants was low enough<sup>271</sup>. Moreover, these autoreactive T cells have a high activation threshold<sup>268</sup>. Bystander activation describes a condition where a viral infection elicits a strong cytokine response in beta-cell surrounding tissue which in turn significantly increases antigen uptake and processing by antigen presenting cells (APCs) such as dendritic cells, B cells, and macrophages which also in such conditions present self determinants. These activated APCs could potentially activate autoreactive T cells since their activation threshold is lowered due to increased amount of APCs with cytokine mediated over expression of costimulator molecules (reviewed in<sup>272</sup>). Autoimmunity induced by bystander activation in mice was supported by other authors<sup>273, 274</sup>. However, according to Von Herrath et al<sup>275</sup> the above description of bystander activation is a classical meaning of the condition; a non-antigen specific, and non-T cell dependent by the TCR, but driven by proinflammatory mediators commonly produced during viral infections. The authors emphasize that most of the responding CD8<sup>+</sup> T cells are not bystanders, but specific for viral epitopes, relevant in mice<sup>276</sup> as well as human<sup>277</sup>. In a TCR-dependent bystander activation cytokines alone are unlikely to drive extensive expansion of T cells in the absence of the TCRs equivalent antigen<sup>278, 279</sup>. The suggested form of activation states; in addition to cytokines, a second signal is required for full activation of the autoreactive cells, and that this signal is connected between the TCR and its equivalent self antigen<sup>275</sup>. They further argue that molecular mimicry and bystander activation can interact. Autoreactive cells are induced by molecular mimicry, but the development of autoimmune disease requires their subsequent activation by bystander means<sup>275</sup>.

## Hygiene hypothesis

Infections may also protect against the development of autoimmune diseases. According to the hygiene hypothesis the exposure to allergens in the early life environment reduces the risk of developing immune disorders<sup>280</sup>. A decreasing incidence in infections due to improved hygiene, vaccination, and use of antibiotics is the main cause of the continual increase of both autoimmune and allergic disease<sup>281</sup>. The model was initially formulated for allergic diseases and the hypothesis was proposed subsequent to the observed inverse correlation between hay fever and the number of older siblings in which 17,414 British children were included<sup>280</sup>. The incidence of allergic diseases has been increasing the last three to four decades and could be explained by environmental factors associated with more industrialized and

urban living. For instance there is a clear difference in allergy prevalence of inhabitants in urban Germany than in farmers of rural Bavaria<sup>282</sup>. The outcome included results from 10,163 children and the conclusion showed that farmers' children had lower prevalence of hay fever, asthma and wheeze than their peers not living in an agricultural environment<sup>282</sup>. The same was interpreted for the 12,876 children in southwest Ethiopia in which it was concluded that all respiratory symptoms were significantly less common overall in the rural area than in the urban population of Jimma Ethiopia<sup>283</sup>. This geographical difference could not be explained by genetic differences, since as shown for type 1 diabetes, children from families having recently immigrated from low-incidence to high-incidence countries develop the disease with high incidence<sup>106, 284, 285</sup>. Furthermore as previously mentioned there was a six-fold gradient difference in the incidence of type 1 diabetes between Russian Karelia and bordering Finland where the predisposing HLA-DQ genotypes are equally frequent in the two populations<sup>35</sup>. The correlation of the decline in infections and the increase in type 1 diabetes is further suggested by observations such as low type 1 diabetes incidence in areas with the highest population density and most household crowding<sup>286</sup> and that the frequency of type 1 diabetes is higher in firstborns of multiplex families than in other children<sup>232</sup>. On the basis of these data, it has been proposed that the lack of infections in industrialized countries; the profound changes in the environmental conditions and in the health care system have resulted in a relative sterilization of the surrounding environment. This reduced exposure of the immune system to antigens in turn has led to an imbalance in immune responses that favours the development of autoimmune diseases such as type 1 diabetes and of allergic and hypersensitivity conditions.

### Cytokines

Cytokines are key mediators in the host response to infection and increased plasma and tissue levels of those mediators are associated with the intensity of the inflammatory response. They are signaling molecules which bind the membrane receptors of immune system cells at fairly low concentrations. Cytokines are small, short-lived polypeptides, approximately 20 kDa in size and characterized by transient production<sup>287</sup>. Cytokines are both pleiotropic (each cytokine may act on several targets) and redundant (numerous cytokines respectively elicit the same cellular response)<sup>288</sup>. Cytokines have been classified to subgroups by their three-dimensional protein structure<sup>289</sup> and by cytokine T helper-1 (Th1) and Th2 CD4<sup>+</sup> T cell effector function<sup>290, 291</sup>. They mediate and regulate immunity, hematopoiesis, trigger inflammation and respond to viral infections (reviewed in<sup>292</sup>). Cytokines operate together in immunological pathways underlying pathogenesis and generally act locally in a paracrine or autocrine manner, but may be endocrine mediators as well<sup>293</sup>.

Cytokines are also important mediators involved in the orchestration of events required for a successful pregnancy<sup>294</sup>. Different hypotheses have been put forward to explain the means by which the fetus avoids maternal rejection<sup>294, 295</sup>, as the precise

mechanisms of the maternal immune response to the fetus are not fully understood. The Th2 paradigm, first published in 1993<sup>294</sup>, proposes a predominance of humoral Th2-type immunity and a decline of cell-mediated Th1-type immunity during a successful pregnancy. The concept of pregnancy as biased toward a Th2 immune response is an oversimplification<sup>296</sup> as cytokines, such as IFN- $\gamma$  and TNF- $\alpha$ , typical Th1 cytokines are necessary during the early stages of pregnancy to support successful implantation and placenta development<sup>297, 298</sup>. However, studies have shown that the levels of IFN- $\gamma$  decrease in maternal plasma after implantation<sup>299</sup> and that circulating IFN- $\gamma$  and IL-1 $\beta$  have been found either absent or in very low levels during the continued normal pregnancy<sup>300, 301</sup>. Higher levels of typical Th1 cytokines after implantation may have deleterious effects on pregnancy outcomes by increasing cell-mediated immunity<sup>302</sup>. For instance, high levels of IFN- $\gamma$  seem to play a critical role in the pathogenesis of unexplained recurrent pregnancy loss<sup>303</sup>. In preeclampsia, a serious complication of the second half of human pregnancy, peripheral blood mononuclear cells synthesize high levels of Th1 cytokines such as IFN- $\gamma$ <sup>304</sup>. As the etiology of disorders such as recurrent pregnancy loss and preeclampsia, remains unknown, it cannot be excluded that virus infections may play an important role since Th1 cytokines serve as markers of current virus infection as well. Since the Th2 paradigm was first published, it has been confirmed<sup>305, 306</sup> as well as weakened by later studies<sup>296, 307</sup>. It is nevertheless considered that a discrete balance between Th1- and Th2-type cytokines is important to maintain a normal pregnancy<sup>295</sup>.

Multiple cytokines and chemokines are released during viral infection as a consequence of pattern recognition receptor signaling in cells such as epithelial cells, macrophages and dendritic cells. Chemokines are of critical importance of coordinating immune cells and cytokines contribute to the activation of both innate and adaptive immunity during viral infection. A virus infection during pregnancy may affect subsets of cytokines<sup>308</sup>. IFN- $\gamma$ , for example, is thought to be essential for elimination of herpes simplex virus infections<sup>308</sup>. This cytokine is produced both in the early stages of infection by natural killer cells and at later stages by activated T cells<sup>309</sup>. IFN- $\gamma$  is a strong activator of macrophages<sup>309</sup> and has multiple mechanisms of viral control, including cell recruitment and activation, polarization of T cell responses and up-regulation of antigen processing and presentation<sup>310</sup>. The antiviral effect of TNF- $\alpha$  is enhanced synergistically with IFN- $\gamma$  in vitro<sup>311</sup>. In turn, interleukin 12 (IL-12), best known for inducing the differentiation of CD4<sup>+</sup> T cells from a Th0 to a Th1 phenotype<sup>312</sup>, is reported to contribute to the regulation of IFN- $\gamma$  production<sup>313</sup>. IL-1 $\beta$ , a potent pyrogen, is also a key mediator of innate immunity, and stimulates neutrophilia and the infiltration of circulating leukocytes into inflamed tissues<sup>314</sup>. It also has an important role in the adaptive immune response by stimulating the development of activated lymphocytes<sup>315</sup>. IL-2 is a T cell growth factor not only produced by CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells and NK cells but also stimulates the proliferation of the lymphocytes mentioned. It enhances cytolytic activity against a variety of target cells and induces the production of other cytokines, including IFN- $\gamma$

and TNF- $\alpha$ <sup>316</sup>. The typical Th2 response is dominated by antibody production and may be seen important in anti-parasite reactions<sup>317</sup>.

Very high blood levels of cytokines may signify hazardous conditions such as sepsis<sup>318</sup>, a state caused by overwhelming inflammatory response towards an infection. For instance IL-6 has been proposed as an important biomarker in sepsis due to its slow and stable plasma kinetics, allowing its easy detection in blood samples, and its good correlation with the intensity of the inflammatory response<sup>318</sup>. However, variations within a physiologic range of multiple cytokines may reflect states of immune dysfunction, immune related disease, or both.

### Celiac Disease

CD is a chronic autoimmune disorder induced by gluten proteins, collectively known as prolamines, present in wheat, barley, and rye. The classical definition of CD includes gastrointestinal manifestations (chronic diarrhea, failure to grow, weight loss, vomiting, abdominal pain and constipation), confirmed by a small bowel biopsy with findings of villous atrophy, crypt hyperplasia, and normalization of the villous architecture which reverts to normal on a gluten-free diet<sup>319</sup>.

During the 1970s the incidence of CD in Swedish children was largely stable<sup>320</sup>. In the mid-1980s a rapid increasing occurrence of CD was observed and within a few years the incidence increased from one to four per 1,000 births<sup>321</sup>. In children below two years of age this increase reached 200–240 cases per 100,000 children. In older children, the incidence remained at a much lower level, although with a slow increase with time which was probably due to improved case finding<sup>322</sup>. This increase in incidence was followed by a sharp decline in 1995 to the previous level of 50–60 cases per 100,000 children<sup>322</sup>. The following analysis of national registry data showed that the explanation of the epidemic was at least in part, a result of change in age at introduction of gluten, amount of gluten given and whether breast-feeding was ongoing or not when gluten was introduced<sup>321</sup>. However, further evidence is required to settle if environmental factors, beyond presence of gluten in the diet, really influence the immunological process resulting in development in CD.

Genetic susceptibility to CD is strongly associated with DQ2 and DQ8. In European populations, more than 90% of patients with CD carry DQ2 which preferentially presents prolamines in the antigen presenting HLA class II heterodimer groove to stimulate intestinal mucosal T cells<sup>323-326</sup>. The pair-wise concordance between monozygotic twins is 75% compared to 11% in dizygotic twins<sup>327</sup>. The major autoantigen is tissue transglutaminase (tTG)<sup>328</sup>, but even though the antigen in CD is identified, the etiology of the disease is not fully understood. For example, T cell reactivity against gluten peptides is clearly demonstrated yet the best predictor of disease is the autoantibodies against tissue transglutaminase. It does seem clear that a combination of genetic and environmental factors is required to develop autoimmunity against tissue transglutaminase and CD<sup>322, 329</sup>.

A previous study indicated that the intrauterine environment, mainly reflected by a low birth weight and neonatal infection diagnosis, could affect the risk of developing CD<sup>330</sup>. An additional study demonstrated that children born in the summer compared with the winter had an increased risk of developing CD reflecting an environmental exposure(s) with a possible seasonal pattern<sup>331</sup>.

Children with type 1 diabetes are at increased risk of CD<sup>332, 333</sup> and may partly be explained by shared HLA in CD and type 1 diabetes<sup>334</sup>. The major risk factor for CD is homozygosity DQ2/2, which has become a disease risk genotype also in type 1 diabetes. In both diseases, genetic susceptibility is associated with the DQ2/8 genotype<sup>41</sup> and it has been proposed that they are pathogenically linked<sup>335</sup>. A recent Italian study of a cohort of 4,322 children and adolescents (age 11.8 ± 4.2 years) identified with type 1 diabetes showed that the prevalence of biopsy-confirmed CD in children and adolescents with type 1 diabetes is high (6.8%); that the risk of having both diseases is threefold higher in children diagnosed with type 1 diabetes at age <four years than in those age >nine years; and that girls have a higher risk of having both diseases than boys<sup>336</sup>. This finding is consistent with the hypothesis that there are common genetic or environmental factors in the etiopathogenesis of these two diseases in younger children.

Two studies in 2003 suggested that early ingestion of cereal or gluten increases risk of type 1 diabetes<sup>249, 250</sup>. The BABYDIAB study follows newborn children (n=1,610) of parents with type 1 diabetes. They concluded that early introduction of gluten-containing foods (before three months of age) was found to be significantly associated with increased risk of islet autoantibody development<sup>249</sup>. Moreover, islet autoantibody risk was not associated with reduced breastfeeding. It can therefore not be excluded that gestational events may sensitize a child to be born with an increased risk for CD. An increased risk might be dependent both on genetic susceptibility and exposure of environmental factors during pregnancy that will increase the risk of autoimmunity.

# Chapter II

## Aims

The primary aim of this thesis was to test whether gestational exposure to environmental factors may induce islet autoimmunity. The second aim was to determine to what extent the exposure to viruses or other environmental factors is a risk factor for type 1 diabetes in the offspring. As children with type 1 diabetes have a higher risk of developing CD, an additional aim was to determine whether markers of possible infections during early pregnancy was associated with development of tissue transglutaminase (tTG) autoantibodies or CD in the offspring.

### Study I

The objective of our first study was to determine seroconversion to islet autoantibodies in non-diabetic mothers during pregnancy.

### Study II

In this study, we collected serum samples that were taken during early pregnancy from mothers who later gave birth to children with confirmed CD before five years of age (n=39) compared to matched controls (n=78). We measured an array of (Th1/Th2) cytokines with the aim of investigating whether CD is already triggered in utero as denoted by quantitative changes in the mother's cytokine profile.

### Study III

In this present study we analyzed cytokines in serum samples collected during early pregnancy from mothers who gave birth to children developing multiple, persistent islet autoantibodies, type 1 diabetes, or both, before seven years of age (n=48). These were compared to controls (n=98) matched for age, sampling date and HLA-DQ genotypes.



## Study IV

Gestational EV infections have been associated with risk HLA-DR as well as with type 1 diabetes in the child. We analyzed EV-RNA and EV-IgM in relation to type 1 diabetes HLA-DQ risk genotypes and to islet autoantibodies in non-diabetic mothers studied both in early pregnancy and at delivery.

### Study design

#### Diabetes Prediction in Skåne

Diabetes Prediction in Skåne (DiPiS), a population based study, obtained blood samples from all mothers in the Skåne (county in southern Sweden) region at the time of delivery between September 2000 and August 2004<sup>337, 338</sup>. The study involves screening of all newborns and aims to identify children in Skåne who are genetically susceptible to type 1 diabetes as well as to monitor if they develop islet autoantibodies and to follow these children for 15 years. A blood sample test kit with a questionnaire was sent to the parents who were asked to bring the child to their Community Health Care Center in order to get a blood sample from their child. This acquired questionnaire was filled out when the child was two months of age with questions regarding their pregnancy such as "Did you have any infections during pregnancy (yes or no)?" Each following year the child will give a blood sample for islet autoantibody analysis. If a child is positive for more than one islet autoantibody, the parents will be contacted and referred for follow up four times per year by a specialist pediatrician in Malmö, Lund, Helsingborg, Kristianstad or Ystad. Approximately 75% of all mothers in Skåne participated in DiPiS. Serum samples were stored at -20 C, thawed and an aliquot removed to be analyzed immediately for islet autoantibodies<sup>245, 339</sup>. The attained cord blood was analyzed for HLA-high risk alleles and the mothers' serum samples for islet autoantibodies: GADA, IA-2A and IAA<sup>124</sup>.

Out of the 48,058 recorded live births, cord blood and serum samples were obtained from 35,827 mothers at the time of delivery (Figure 3). The selection criteria for islet autoantibody positive mothers at delivery have been detailed elsewhere<sup>245</sup>. A total of 2001 mothers were excluded because they suffered from diabetes (gestational, type 1, type 2, unknown type or uncertain diagnosis). The delivery sample from the non-diabetic mothers was also analyzed once the cord blood was positive for any of the three islet autoantibodies<sup>245, 339</sup>. A total of 532 mothers gave birth to children with cord blood islet autoantibodies (Figure 3). Due to the fact that mothers' serum sample at delivery was missing from 103 mothers, a total of 429 islet autoantibody positive mothers were available. For every islet autoantibody positive mother, four islet autoantibody negative mothers were selected (n=1,716); three of these were randomly selected matched by the year of birth and sampling date ( $\pm$  seven days). One mother was randomly selected matched by the year of birth. Of the 429 remaining islet autoantibody positive mothers, 187 early pregnancy samples were missing in the

Southern Sweden Microbiological biobank (SSM-biobank). These 187 mothers were lost at random and did not differ from the 429 mothers with respect to age, gestational length or parity. Hence, it was possible to analyze a total of 242 early pregnancy samples (gestational week 10-16) from islet autoantibody positive mothers (Figure 3). Additional early pregnancy serum samples were obtained from 48 mothers giving birth to children developing multiple, persistent islet autoantibodies, type 1 diabetes, or both before seven years of age (Figure 3). This group of mothers initially included 53 mothers, however four were excluded because of preeclampsia (n=2) and gestational age at birth was less than 256 days (n=2). In addition one mother was excluded because both her matched controls had preeclampsia. A further group included in this thesis refers to early pregnancy serum samples from obtained from 39 mothers giving birth to children who later developed high titers of tTGA, CD, or both before 5 years of age (Figure 3). At first this group of mothers included 50 mothers, however as some mothers suffered from pregnancy complications such as preeclampsia and hypertension they were excluded.

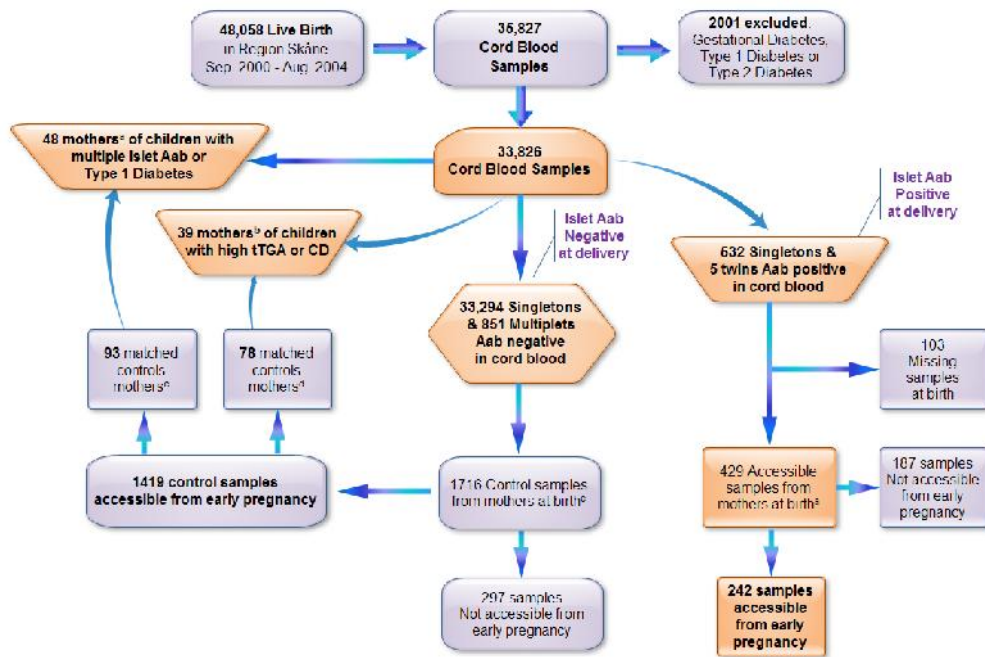


Figure 3. Study populations in this thesis.

<sup>a</sup> Refers to early pregnancy serum samples obtained from mothers giving birth to children developing multiple, persistent islet autoantibodies, type 1 diabetes, or both before seven years of age.

<sup>b</sup> Refers to early pregnancy serum samples obtained from mothers giving birth to children who later developed high titers of tTG antibodies, celiac disease, or both before five years of age

<sup>c</sup> Matched to the 48 mothers for age, sampling date and HLA-DQ genotypes

<sup>d</sup> Matched to the 39 mothers for age, sampling date and HLA-DQ genotypes

<sup>e</sup> All samples are serum samples from mothers to singletons.

## Celiac Disease Prediction in Skåne

The prospective cohort study CiPiS (Celiac Disease Prediction in Skåne) is part of the DiPiS<sup>338</sup> which aims to determine the etiology indicators of CD in newborn children in the region of Skåne born between June 2001 and August 2004<sup>340</sup>. Children with CD HLA-risk alleles were identified and asked to participate. When HLA-risk alleles were recognized, included children in CiPiS were screened for tTGA. CD was established by intestinal biopsy in children with a confirmed positive tTGA test.

## The Southern Sweden Microbiological biobank

Early pregnancy serum samples (gestational week 10-16) was retrieved from the SSM-Biobank which contains more than 120,000 stored plasma samples (public health test), obtained from all pregnant mothers at their first visit to the Maternity Care Center between 1986 and 2006. The serum samples from this public health test were used to screen for antibodies against rubella, hepatitis B, HIV and syphilis. Leftover serum samples were stored at -20°C in the SSM-biobank<sup>341</sup>. The SSM-biobank stores all samples submitted for microbiological diagnosis in Southern Sweden as well<sup>341</sup>.

All clinical data was retrieved from the Regional perinatal registry (Perinatal Revision South), which contains perinatal data from all deliveries in the Southern Health Care Region of Sweden.

# Chapter III

## Methodology

The mothers (n=1,822) included in this thesis were analyzed for HLA genotype, islet antibodies (GADA, IA-2A and IAA in both early pregnancy samples and at delivery), ten different cytokines (early pregnancy samples) and IgM class EV antibodies (early pregnancy samples).

### HLA-typing

HLA-typing is described elsewhere <sup>342, 343</sup>. In brief, HLA was analyzed on dried blood spots (DBS) on 3 mm in diameter filters and punched into 96-well PCR plates. Following PCR, the amplified biotin conjugated product was transferred to DELFIA streptavidin-coated microtitration plates (Perkin Elmer Life Sciences, Boston, MA, USA). HLA-DQ alleles were analyzed by hybridization with sequence-specific oligo nucleotide probes, conjugated with samarium, europium or terbium. The fluorescence results were read through a VICTOR2 MultiLabel Counter (Perkin-Elmer Life Sciences, Inc, USA).

### Autoantibody analysis

GADA and IA-2A antibody titers were determined in radioligand binding assays described previously <sup>89</sup>. Briefly, to generate the recombinant antigens for GADA and IA-2A respectively, *in vitro* transcription/translation was used. During these steps the antigens are made radioactive by <sup>35</sup>S-methionine incorporation. The antigen was directly immunoprecipitated with human serum and the antibody-bound labeled antigen was separated from free by Protein A-Sepharose (Amersham Biosciences, Uppsala, Sweden). The radioactivity was counted in a Beta Plate Reader (Perkin Elmer Life Sciences, Waltham, Massachusetts, USA) as described elsewhere <sup>344</sup>. The titers of islet autoantibodies in the early pregnancy samples were determined from the international WHO standard for GADA and IA-2A, which defines titers in Units/mL (U/mL) <sup>345</sup>. Our laboratory is participating in the Diabetes Autoantibody Standardization Program (DASP) <sup>346</sup>. In the DASP 2009 workshop our workshop specificity and sensitivity for GADA was 68% and 99%, respectively and for IA-2A it was 60% and 99%, respectively.

IAA titers were determined in a competitive radioligand assay previously described<sup>345</sup>. In brief, in the IAA assay recombinant insulin (Perkin Elmer Life Sciences) was labeled with <sup>125</sup>I. Antibody-bound and free-labeled insulin were separated with Protein A-Sepharose (Zymed, San Francisco, CA, USA). The competitive assay was performed using unlabelled human insulin (Actrapid, Novo Nordisk, Bagsvaerd, Denmark). Results were expressed in arbitrary units. The DASP workshop sensitivity and specificity for IAA was 20% and 89%, respectively.

#### Cytokine measurements

All serum samples were tested using a multiplex sandwich enzyme-linked immunosorbent (Th1/Th2) ultra sensitive assay (Mesoscale, Gaithersburg, Maryland), in which every cytokine was measured at the same time in one single sample (5 µL human serum). In the assay, the following cytokines were measured; IFN- $\gamma$ , IL-10, IL-12p70, IL-13, IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-8 (chemokine) and TNF- $\alpha$ , as per the manufacturer's protocol on a SECTOR 6000 instrument (<http://www.mesoscale.com>). The same operator performed all the cytokine measurements. During processing the operator was blinded as to the origin of the samples.

#### Real-time RT-PCR (rRT-PCR)

Enteroviral genome detection was carried out after RNA was extracted from 100 µL serum specimens using MagNA Pure LC RNA Isolation Kit - High Performance, Roche Applied Science (Roche Applied Science, Indianapolis, IN, USA)<sup>347</sup>. Use of automated MagNA Pure sample processing reduces the hands-on time required to process specimens. It also minimizes specimen-to-specimen contamination during the automated processing.

The occurrence of EV-RNA was detected by rRT-PCR assay detecting as few as ten RNA copies per reaction<sup>348, 349</sup>. The rRT-PCR primers and probes target sites in the 5'-non-translated region which is fully conserved among all EVs and allows amplification of all members of the genus<sup>350</sup>. The method is intended for use in the WHO Global Polio Laboratory Network for rapid and large-scale identification of poliovirus field isolates.

#### Capture IgM ELISA

Capture IgM ELISA is described elsewhere<sup>187</sup>. Briefly, IgM class EV antibodies were measured against a mixture of three EV antigens (CBV 3, CAV 16 and echovirus 11) using a capture EIA method. Microtiter plates (Nunc-Immuno<sup>TM</sup> MaxiSorp, Nunc, Glostrup, Denmark) were coated with monoclonal anti-human IgM antibodies

(Medix Biochemica, Kauniainen, Finland) and serum was incubated for 1 h at 37°C in PBS supplemented with BSA, NaCl and Tween 20. The mixture of heat-treated EV antigens (10 µg/ml each) was incubated for 30 min at 37°C, after which a comparable mixture of biotinylated detection antibodies (10 µg/mL each) was added and incubated. Streptavidin-horseradish peroxidase conjugate (Life Technologies, Gaithersburg, MD) was incubated and orto-phenylenediamine-dihydrochloride substrate was added. Color reaction was measured at 490 nm. The cut-off level of seropositivity was determined to be three times the level of conjugate controls included in each assay. The detection antibodies were produced by immunizing rabbits by purified heat-treated CBV 3, CAV 16 and echovirus 11. The IgG fraction of rabbit hyperimmune sera was purified in a fast protein liquid chromatography using a protein A column (Pharmacia Fine Chemicals, Uppsala, Sweden), and biotinylated.

## Statistics

### Paper I

Data were analyzed using standard software SPSS (version 16.0 [www.spss.com](http://www.spss.com)). GADA, IA-2A and IAA titers were not normally distributed and were therefore logarithmically transformed. Differences in titers of islet autoantibodies between control- and case mothers were assessed by unpaired Student t-test.

### Paper II

The cytokines were first examined as continuous variables with  $\log_{10}$  base transformation made to normalize the cytokine measurements. A Mann-Whitney U test tested for a significant overall shift in cytokine levels in cases compared to controls showing the median and interquartile (IQR) range. To show an actual change in mothers' cytokine profile, it is thus necessary to control for the correlation within the groups. We therefore investigated the association of the cytokines using the Spearman correlation coefficient to examine the association between cytokines levels both among the cases and controls. To account for the matching design of the study a conditional logistic regression model was applied to examine the association of each cytokine with CD. The odds ratios and 95% confidence intervals are also reported.

### Paper III

Statistical analyses were performed using Software SPSS (version 16.0 [www.spss.com](http://www.spss.com)) and R 2.12.1 ([www.r-project.org](http://www.r-project.org)). Chi-squared and Fisher exact tests were used to compare the baseline characteristics of the index and control mothers. Cytokine levels were not normally distributed hence the Mann-Whitney U-test was used to compare cytokine levels in the index and control mothers. Spearman rank coefficient of correlation was used to determine the strength and direction of correlation between all pairs of cytokines. In order to account for the matching between index and control mothers, we performed conditional logistic regression with the group indicator (1 =

index, 0 = control) as the outcome and log (base 2) transformed cytokine level as the main effect, adjusting for gestational length and parity in the adjusted analysis. One model was fit for each cytokine.

#### Paper IV

Differences in proportions across categorical groups were tested using chi-square tests. Simple logistic regression models were fitted to examine separately whether 1) the distributions of HLA-DQ genotypes conferring risk for type 1 diabetes and 2) EV-IgM differed in the islet autoantibody positive mothers in early pregnancy and at delivery compared to controls. Multiple logistic regression models were next fitted with both HLA-DQ risk genotypes and EV-IgM in the model to examine whether these factors were independently associated with islet autoantibodies both before and after adjusting for maternal age, parity, year of birth and season-quarter of early pregnancy. The final model included a HLA-DQ\*EV-IgM interaction term to test for gene-environment interaction. P-values less than 0.05 were considered statistical significant



# Chapter IV

## Results

### Paper I

#### Occurrence of single islet autoantibodies

GADA: The frequency of mothers positive for GADA both in early pregnancy and at delivery was 92% (123/133) indicating that 8% (10/133) seroconverted to GADA during pregnancy (Table 4). The GADA titers (U/mL) in these mothers increased significantly between early pregnancy and delivery corresponding to a mean increase of 409% ( $p < 0.0001$ ) (data not shown). In the remaining group of 123 mothers already positive during early pregnancy only 4% (5/123) showed a minor increase in GADA titers (U/mL). The difference in titers was not statistically significant (Table 4). The major group, 96% (118/123), showed a significant decrease in GADA titers during pregnancy ( $p < 0.0001$ ). The GADA titers (U/mL) in these mothers decreased significantly between early pregnancy and delivery corresponding to a mean decrease of 69% ( $p < 0.0001$ ) (data not shown).

IA-2A: There were three (3/19) mothers who seroconverted to IA-2A between early pregnancy and delivery. Seroconversion in IA-2A titers (U/mL) were low and close to the cut-off limit at delivery. The difference in titers in these three mothers was not statistically significant (Table 4). Similar to GADA, a majority, 84% (16/19) showed a significant decrease in IA-2A titers during pregnancy ( $p < 0.0001$ ). The IA-2A titers (U/mL) in these mothers decreased significantly corresponding to a mean decrease of 83% ( $p < 0.0001$ ) (data not shown).

IAA: The frequency of IAA positive mothers both at early pregnancy and at delivery was 65% (69/106) indicating that as many as 35% (37/106) of the mothers seroconverted to IAA during pregnancy (Table 4). The IAA titers (RU) in these mothers increased significantly corresponding to a mean increase of 1962% ( $p < 0.0001$ ) (data not shown). Of the remaining 69 mothers already IAA positive in early pregnancy, 36% (25/69) showed a significant increase in titers (RU) between early pregnancy and delivery ( $p < 0.0001$ ). The mean increase amounted to 192% ( $p < 0.0001$ ) (data not shown). As many as 44/69 (64%) of the mothers with IAA during early pregnancy demonstrated a decrease in IAA titers (RU) between the early pregnancy and delivery. The mean decrease was 39% ( $p < 0.0001$ ) (data not shown).

Table 4. Single autoantibody distribution of GADA (n 133), IA-2A (n 19) and IAA (n 106) at early pregnancy and at the time of delivery in islet autoantibody positive mothers and controls.

	GADA	IA-2A	IAA
	n/Total (%)	n/Total (%)	n/Total (%)
Mothers autoantibody positive at delivery	133/242 (55)	19/242 (8)	106 (44)
Mothers autoantibody positive at early pregnancy	123/133 (92)	16/19 (84)	69/106 (65)
Seroconverting mothers	10/133 (8)	3/19 (16)	37/106 (35)
Difference in titer (mean + SEM)*	0.66 ± 0.08	0.32 ± 0.11	0.52 ± 0.05
p value	<0.0001	0.107	<0.0001
Mothers with increased autoantibodies	5/123 (4)	-	25/69 (36)
Difference in titer (mean + SEM)*	0.15 ± 0.10	-	0.23 ± 0.04
p value	0.217	-	<0.0001
Mothers with decreased autoantibodies	118/123 (96)	16/16 (100)	44/69 (64)
Difference in titer (mean + SEM)*	-0.60 ± 0.03	- 0.85 ± 0.086	-0.25 ± 0.03
p value	<0.0001	<0.0001	<0.0001
Control mothers at delivery	0/1419 (0)	0/1419 (0)	0/1419 (0)
Control mothers at early pregnancy	15/1419 (1)	3/1419 (0.2)	4/1419 (0.3)

\* Mean difference in titer (mean + SEM)\* between early pregnancy and delivery is shown in U/mL for GADA and IA-2A and in RU for IAA.

#### Occurrence of multiple islet autoantibodies

We next analyzed mothers with multiple islet autoantibodies comparing early pregnancy with delivery. One mother had GADA and IAA both at early pregnancy and at delivery. Of the 13 mothers with both GADA and IA-2A at delivery, only two turned double positive as they both developed IA-2A during pregnancy. Only one mother seroconverted to triple positivity at delivery. As she was IAA positive at early pregnancy, she developed both GADA and IA-2A. None of the controls had multiple autoantibodies during early pregnancy.

## Paper II

All ten cytokines in cases were plotted against controls on a logarithmic scale in box plots (Figure 4), showing the median value of cytokines within the IQR to illustrate the probable distribution of the different cytokines between cases and the matched controls. Increased levels for TNF- $\alpha$  ( $p=0.001$ ), IL-13 ( $p=0.004$ ) and IFN- $\gamma$  ( $p=0.003$ ) was observed in cases as compared to controls. Elevated levels among cases were also observed for IL-10 ( $p=0.027$ ), IL-2 ( $p=0.02$ ), IL-1 $\beta$  ( $p=0.012$ ) and IL-12 ( $p=0.045$ ).

To account for the matching design of the study we performed a logistic regression. The odds ratio in a 95% CI interval showed statistical significance for the cytokines IFN- $\gamma$  (OR 4.92;  $p=0.005$ ), IL-13 (OR 2.27;  $p=0.013$ ), IL-1 $\beta$  (OR 3.13;  $p=0.016$ ), IL-2 (OR 1.91,  $p=0.034$ ), and IL-10 (OR 1.86;  $p=0.045$ , thereby supporting the initial non-parametric analysis (data not shown).

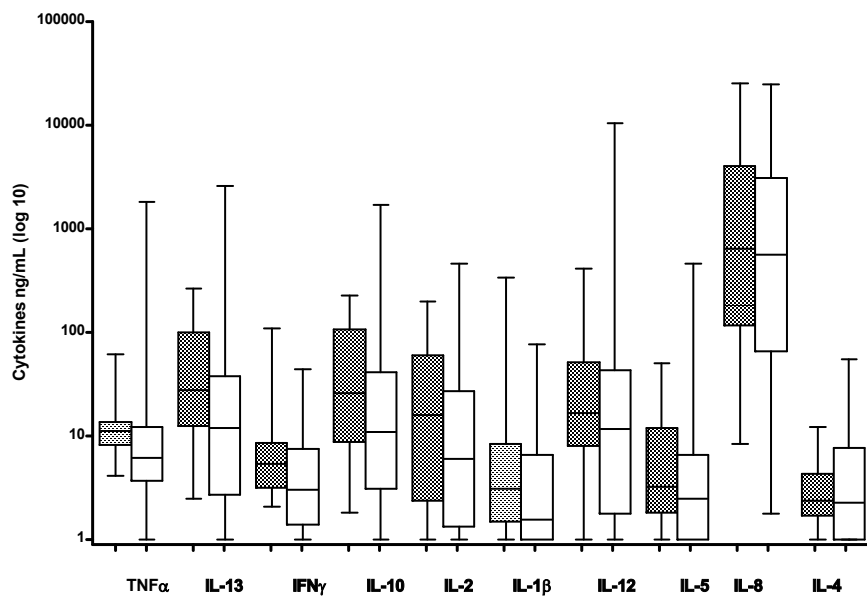


Figure 4. Logarithmic scale box plot of analyzed cytokines in mothers giving birth to children who had elevated levels of tTG and/or an intestinal biopsy confirming CD before five years of age (filled boxes) and in matched controls (empty boxes). The boxes show medians and upper and lower quartiles of the data, while the whiskers indicate the minimum and maximum values. (From reference <sup>351</sup>).

### Paper III

The cytokine levels were higher in the index than in the control mothers. This included IFN- $\gamma$  ( $p=0.02$ ) and IL-1 $\beta$  ( $p=0.04$ ). The odds of multiple, persistent islet autoantibodies, type 1 diabetes, or both, in the offspring increased 1.39-fold (1.04-1.85) with every two-fold increase in IFN- $\gamma$  (adjusted) (Table 5). There was weak evidence to suggest that odds of multiple, persistent islet autoantibodies, type 1 diabetes, or both, in the offspring increased 1.22-fold (1.01-1.48) with every two-fold increase in IL-2, however after adjusting for parity and gestational length the association was no longer significant (Table 5).

A significant correlation was observed between all cytokines ( $p<0.0001$ ), except for IL-4, both within the index and within the control group (data not shown). The observed correlation between the cytokines amongst the control mothers had similar  $r$ -values and associations as detected in the index mothers.

Table 5. Increased cytokine levels during early pregnancy and the risk of multiple, persistent islet autoantibodies, type 1 diabetes, or both, before seven years of age.

Cytokine	Unadjusted		Adjusted*	
	OR (95% CI)	p-value	OR (95% CI)	p-value
IFN- $\gamma$	1.42 (1.07-1.88)	0.015	1.39 (1.04-1.85)	0.026
IL-2	1.22 (1.01-1.48)	0.042	1.21 (0.99-1.48)	0.057
IL-4	1.24 (0.93-1.66)	0.141	1.28 (0.95-1.72)	0.108
IL-1 $\beta$	1.21 (0.97-1.5)	0.085	1.19 (0.95-1.48)	0.126
IL-8	1.09 (0.98-1.22)	0.125	1.09 (0.97-1.22)	0.158
IL-13	1.19 (0.97-1.46)	0.091	1.16 (0.94-1.43)	0.170
IL-5	1.17 (0.95-1.44)	0.146	1.13 (0.91-1.4)	0.254
IL-10	1.12 (0.93-1.35)	0.221	1.1 (0.91-1.33)	0.340
TNF- $\alpha$	1.11 (0.91-1.34)	0.301	1.09 (0.89-1.33)	0.424
IL-12	1.04 (0.90-1.2)	0.586	1.0 (0.85-1.17)	0.982

The odds ratios (OR) and 95% confidence interval (95% CI) in a conditional logistic regression analysis to test whether cytokine levels (twice or more than the controls) during early pregnancy were associated with an increased risk for multiple, persistent islet autoantibodies, type 1 diabetes, or both, before seven years of age. \* Adjusted for gestational age and parity.

Paper IV

The frequency of islet autoantibody positive mothers with EV-IgM in early pregnancy 12% (44/365) was not statistically different from controls 11% (156/1457) ( $p=n.s.$ ).

We next examined the distribution of HLA-DQ genotypes conferring risk for type 1 diabetes among the group of non-diabetic mothers at delivery. It was found that the DQ2/2, 2/X risk genotypes were more frequent among islet autoantibody positive mothers compared to the controls (Table 1). We therefore analyzed whether the frequency of mothers with EV-IgM in early pregnancy differed by type 1 diabetes HLA-DQ risk genotype. Among the mothers who were islet autoantibody negative at the delivery, the frequency of EV-IgM did not differ between the type 1 diabetes risk genotypes (data not shown). Among the mothers positive for islet autoantibodies at delivery, the percentage with EV-IgM differed significantly across HLA-DQ risk groups ( $p=0.022$ ) (data not shown).

Our further analysis demonstrated that mothers with DQ2/2, 2/X were significantly more likely to have had EV-IgM in early pregnancy (22%) compared to other islet autoantibody positive mothers (9%;  $p<0.001$ ). This difference was seen both among mothers who seroconverted from early pregnancy (25% compared to 9%,  $p=0.012$ ) and among mothers who were islet autoantibody positive already in early pregnancy (19% compared to 8%,  $p=0.038$ ) (data not shown).

When comparing all islet autoantibody positive mothers at delivery with controls, a gene-environment interaction of HLA-DQ2 and EV-IgM was associated with presence of islet autoantibodies at delivery ( $p=0.008$ ) after adjusting for parity, maternal age, year of birth and season of sample in the early pregnancy (Table 7).

Table 6. HLA-DQ T1D risk genotype distribution among mothers positive for islet autoantibodies at delivery and controls

	Controls n (%)	Ab + n (%)	OR	Controls V's Ab+ 95%CI	p-value
HLA-DQ risk genotypes					
Non T1D risk (DQX/X)	592 (42.7)	128 (36.8)	1.00	ref	
Very high risk (DQ2/8)	44 (3.2)	12 (3.4)	1.26	0.65 – 2.45	0.495
High risk (DQ8/8, 8/X)	223 (16.1)	63 (18.1)	1.31	0.93 – 1.83	0.122
Moderate risk (DQ2/2, 2/X)	208 (15.0)	83 (23.9)	1.85	1.34 – 2.54	<0.001
Low risk (DQ6.2)	321 (23.1)	62 (17.8)	0.89	0.64 – 1.25	0.506

Table 7. Association of having a HLA-DQ2 genotype and EV-IgM in the early pregnancy on the presence of islet autoantibodies at delivery

Factors	Unadjusted			Adjusted *		
	Odds ratios of islet autoantibodies in cord blood			Odds ratios of islet autoantibodies in cord blood		
	OR	95%CI	p-value	OR	95%CI	p-value
HLA-DQ2 risk genotype						
No	1.00	ref		1.00	ref	
Yes (DQ2/2, 2/X)	1.52	1.11 – 2.09	0.009	1.55	1.13 – 2.13	0.009
EV-IgM positive						
no	1.00	Ref		1.00	Ref	
yes	0.76	0.48 – 1.21	0.249	0.74	0.46 – 1.18	0.206
HLA-DQ2 * EV-IgM pos						
DQ2/2, 2/X and EV-IgM pos	2.93	1.29 – 6.66	0.010	3.10	1.35 – 7.15	0.008

\*Multiple logistic regression adjusting for parity, maternal age, year of birth and season of early pregnancy (quarters)

# Chapter V

## Discussion

A major finding of the present investigations was that islet autoantibodies more often than expected were already present during early pregnancy in the majority of mothers who at delivery were islet autoantibody positive. Our first question was therefore whether non-diabetic pregnant women who were islet autoantibody positive at the time of delivery developed these autoantibodies during pregnancy, or if they already had islet autoantibodies in early pregnancy. By contrasting the islet autoantibody titers in early pregnancy with that of delivery, three patterns were detected. The dominant pattern was that mothers who had islet autoantibodies in early pregnancy showed a significant and easily detectable decrease in titers over the course of pregnancy. This phenomenon is similar to other autoimmune diseases<sup>352, 353</sup>. The second pattern was found in a subgroup of mothers, who showed an increase in islet autoantibodies. The third pattern was in mothers who developed GADA, IA-2A or IAA. These mothers represent a subgroup of mothers who seroconverted during pregnancy. The detection of seroconversion was directly relevant to the main finding in the second study<sup>351</sup> where several cytokines were increased in early pregnancy in mothers who gave birth to children developing CD before five years of age. It was concluded from this observation that mothers who experience an increase in seven out of ten cytokines tested may have had an infection or otherwise had been exposed to environmental factors that allowed their cytokine pattern to be different from matched controls. Exposures of factors that increase the levels of certain cytokines may therefore represent a trigger phenomenon that is associated with an increased risk for the fetus to develop CD after birth. This observation prompted us to analyze serum samples from early pregnancy for the same cytokines but this time in serum samples from mothers who had given birth to children who developed multiple, persistent islet autoantibodies, type 1 diabetes, or both, before seven years of age.

The finding showed that at least two Th1 cytokines were increased in early pregnancy in these mothers. The pattern of cytokines was different from that of mothers who gave birth to a child developing CD. Nevertheless, it was possible to conclude that mothers who gave birth to a child with persistent islet autoantibodies, type 1 diabetes, or both, before seven years of age may have had an infection or been exposed to another event that increased IFN- $\gamma$  and IL-2 in early pregnancy.

The last finding was that EV-IgM in early pregnancy was strongly associated with mothers who had type 1 diabetes high risk HLA-DQ. However, while there was no significant association between EV-IgM and islet autoantibodies in early pregnancy,

seroconversion was associated with EV-IgM during early pregnancy. Taken together, these four studies support the view that gestational events, most likely related to infections, may contribute to the risk for a child to develop either type 1 diabetes or CD.

The present investigation has several strengths. The first was the availability of mothers participating in the DiPiS study<sup>124, 339</sup>. These mothers represent 75% of all mothers who gave birth in Skåne in the years between 2000 and 2004 and may therefore qualify as a population-based study. Very few mothers did decline to participate at the time of delivery. It is also important to note that mothers with type 1 or 2 diabetes as well as gestational diabetes were also participating in DiPiS. However, as any form of diabetes will compromise the fetus all these mothers were excluded in the present investigation. Other authors have studied DiPiS mothers with gestational diabetes<sup>354</sup>. Large cohort studies such as BABYDIAB<sup>128, 185</sup> and DAISY<sup>123, 355</sup> have provided extensive information about children born to type 1 diabetes mothers. A second strength was the availability of serum samples collected during early pregnancy from the SSM-Biobank which contains more than 120,000 stored plasma samples (public health test), obtained from all pregnant mothers at their first visit to the Maternity Care Center between 1986 and 2006. It was possible to identify the DiPiS mothers in the SSM-Biobank and thereby obtain the relevant stored early pregnancy samples of these mothers. Furthermore, once the early pregnancy samples were obtained it was possible to retrieve clinical data from the Regional perinatal registry (Perinatal Revision South) to correct for gestational events including preeclampsia and high blood pressure. A final strength to the study population was the possibility to obtain well-matched control mothers.

The validation of the islet autoantibodies is a further strength. Our laboratory has long been involved in an international effort to standardize islet autoantibodies<sup>356</sup> starting with the Immunology of Diabetes Workshop (IDW) effort. The IDW was eventually replaced by the Diabetes Autoantibody Standardization Program (DASP)<sup>346</sup>. During the course of the present investigation our laboratory participated in at least three DASP workshops with analyses of GADA, IA-2A and IAA. For example, forty-six laboratories in 13 countries concluded that the workshop sensitivity of IA-2A in type 1 diabetes study was 58%, less than GADA but higher than IAA<sup>105</sup>. In the DASP 2009 workshop our workshop specificity and sensitivity for GADA was 68% and 99%, respectively and for IA-2A it was 60% and 99%, respectively. In the DASP 2009 workshop our workshop specificity and sensitivity for IAA was 20% and 89%, respectively. It is important to note that the DASP workshops do not define diagnostic sensitivity and specificity as the samples collected for the workshops are defined by volume rather than being representative for new onset patients.

A number of companies provide multiplex type assays for serum or plasma cytokines. The validation of the Mesoscale method used in this thesis found that the Mesoscale was potentially useful in analysis of clinical samples<sup>357</sup>. This method effectively allows multiple analytes to be analyzed simultaneously from small volume samples (5–100  $\mu$ L/test). In terms of performance, the Mesoscale was found to be more sensitive and with the lowest level of quantification being lower than the Biosource (Invitrogen)



Luminex kit for all the cytokines related to the ultra sensitive Th1/Th2 kit <sup>357</sup>. The results from this study also showed that both methods had comparable accuracy when measuring high concentration cytokines but at low concentration the accuracy was improved by the use of the Mesoscale.

Analyses of cytokines are complicated by the fact that cells may produce more than one type of cytokine. Serum cytokines therefore represents the sum of cytokines produced by one or several cell types. We therefore carried out a Spearman rank correlation coefficient (rho) matrix <sup>358</sup> comparing the strength and direction of correlation between all the cytokines and the chemokine which was used to validate the Mesoscale ultrasensitive kit (Tables 8 and 9).

Table 8. Correlation between cytokines among the cases (n=48)

	IFN- $\gamma$	IL-10	IL-12	IL-13	IL-1 $\beta$	IL-2	IL-4	IL-5	IL-8	TNF- $\alpha$
IFN- $\gamma$	1									
IL-10	0.53	1								
IL-12	0.52	0.72	1							
IL-13	0.51	0.89	0.70	1						
IL-1 $\beta$	0.58	0.84	0.52	0.85	1					
IL-2	0.63	0.86	0.54	0.82	0.89	1				
IL-4	0.69	0.29	0.23	0.31	0.37	0.41	1			
IL-5	0.59	0.78	0.71	0.79	0.75	0.79	0.39	1		
IL-8	0.54	0.84	0.58	0.83	0.81	0.89	0.34	0.81	1	
TNF- $\alpha$	0.64	0.76	0.58	0.78	0.82	0.76	0.31	0.71	0.67	1

The correlation coefficients between the cytokines in the index group (n=48) varied between 0.23-0.89 for a total of 45 correlations (all p-values were <0.0001) (Table 8). Exceptions were the correlations between IL-4 and IL-10 (r=0.29; p=0.042), IL-4 and IL-12 (r= 0.23; p=0.097), IL-4 and IL-13 (r= 0.31; p=0.028), IL-4 and IL-1 $\beta$  (r=0.37; p=0.007), IL-4 and IL-2 (r= 0.41; p=0.003), IL-4 and IL-5 (r= 0.39; p=0.004), IL-4 and IL-8 (r= 0.34; p=0.014) and IL-4 and TNF- $\alpha$  (r= 0.31; p=0.028).

Table 9. Correlation between cytokines among the controls (n=93)

	IFN- $\gamma$	IL-10	IL-12	IL-13	IL-1 $\beta$	IL-2	IL-4	IL-5	IL-8	TNF- $\alpha$
IFN- $\gamma$	1									
IL-10	0.69	1								
IL-12	0.69	0.77	1							
IL-13	0.68	0.87	0.83	1						
IL-1 $\beta$	0.66	0.82	0.69	0.80	1					
IL-2	0.71	0.81	0.63	0.77	0.82	1				
IL-4	0.51	0.21	0.28	0.25	0.17	0.36	1			
IL-5	0.76	0.86	0.87	0.84	0.83	0.78	0.29	1		
IL-8	0.65	0.82	0.66	0.82	0.82	0.87	0.22	0.80	1	
TNF- $\alpha$	0.68	0.78	0.69	0.81	0.77	0.72	0.24	0.77	0.74	1

The correlation coefficients between the cytokines in the control group (n=93) (Table 9) varied between 0.17-0.87 for a total of 45 correlations and all p-values <0.0001 except for the correlation between IL-4 and IL-10 (r= 0.21; p=0.037), IL-4 and IL-12 (r=0.28; p=0.004), IL-4 and IL-13 (r= 0.25; p=0.011), IL-4 and IL-1 $\beta$  (r= 0.17; p=0.091), IL-4 and IL-5 (r= 0.29; p=0.003), IL-4 and IL-8 (r= 0.22; p=0.030) and IL-4 and TNF- $\alpha$  (r= 0.24; p=0.016).

We conclude from these validation analyses of both index and control mothers that all the cytokines with a possible exception of IL-4 are well correlated and may be coordinately expressed.

A final strength to our study is the use of the EV-IgM assay as carried out by Hyöty et al.<sup>184, 187</sup>. This assay has been used in a number of key investigations on the possible importance of EV infections in relation to type 1 diabetes<sup>184, 187</sup>.

Possible limitations to the present study include the potential change in cytokine levels associated with blood sample handling. The above validation showed significant correlation between all cytokines with a possible exception of IL-4. However, the poor correlation with IL-4 may be due to the fact that this cytokine is thought to be difficult to measure. If IL-4 may be more difficult to measure it may result in a weaker correlation. There are contradictory reports as to the effect on serum or plasma levels associated with leaving samples at room temperature for a longer period of time. One study showed that storing whole blood samples at room temperature resulted in a

decline in IL-6 but an increase in TNF- $\alpha$  after 4 h<sup>359</sup>, while another study that used whole blood spiked with human recombinant cytokines reported significant declines after 24 h in TNF- $\alpha$  and IL-6, but not IFN- $\gamma$ <sup>360</sup>. An additional study revealed that cytokines were quite stable if refrigerated or frozen. However, samples kept at room temperature for 20 days, had lower TNF- $\alpha$  levels than the mean values in samples kept at 4 and -70°C. Plasma and serum cytokine and soluble marker levels were comparable<sup>361</sup>.

Another possible limitation of our study was that we did not have access to the BMI of the mothers during early pregnancy. It has been reported that non-pregnant obese women have elevated levels IL-6<sup>362</sup>.

Previously, the role of EV infections has been evaluated in case-control studies showing higher frequencies in patients with newly diagnosed type 1 diabetes than in healthy control subjects of EV antibodies<sup>197, 201, 205, 363</sup> and, more recently, EV genome<sup>208, 209, 210, 216</sup>. As can be observed in Tables 2a – 2c, studies on associations between EV infections and type 1 diabetes at the clinical onset (Table 2c) are much more common and several studies showed strong associations. However, these studies are rather revealing virus infections that may accelerate the clinical onset than triggering seroconversion to islet autoimmunity (Table 2b).

Several of the previously conducted studies on the associations between EV infections and type 1 diabetes have failed to match for genetic susceptibility and other confounding factors such as sampling date and age. They show lack of sufficient study size, failure to assess exposure at very early stage or the inability to follow a sufficient number of children long-term. To address these issues a large group of children need to be followed prospectively from birth, with collection of appropriate samples at frequent intervals. The TEDDY study, a consortium of six international centres using a commonly designed protocol has a vast opportunity to identify environmental factors which may contribute to the risk of type 1 diabetes. This large, uniform prospective study follows children's diet, illnesses, allergies and other life experiences. Blood samples are drawn every three months for four years. After four years, children will be seen every six months until the child is 15 years old. One drawback with the TEDDY study is that little information will be available about the possible role of gestational infections in type 1 diabetes in the offspring.

The data generated in this thesis has made it possible to uncover gestational events which may trigger the risk for islet autoimmunity or CD in the fetus. The immune system during fetal life is immature<sup>122</sup> and may therefore be vulnerable to a variety of immunological triggers mentioned throughout this thesis. We believe that it will be of critical importance in the future to investigate pregnant women (index mothers) and to follow not only these women more frequently but their offspring as well. The sample interval should be no longer than two – three months. The following index mothers should be included:

- index mothers who report infectious episodes or psychological stress during early pregnancy i.e. prior to 11-14<sup>th</sup> week of gestation
- who actively report gestational infections from 14-25<sup>th</sup> week of gestation.
- who were islet autoantibody positive in early pregnancy (identified after 14<sup>th</sup> week of gestation)
- control mothers matched for HLA genotype, age, seasonal variation and parity.

In a future prospective study, it would be possible to identify index mothers defined as having gestational infections, psychological stress (e.g. life events), or both a) prior to 11-14<sup>th</sup> week of gestation and b) during the 14-25<sup>th</sup> week of gestation. A study like this would make it possible to determine whether infections, psychological stress including severe life events, or both, alter the profiles of gestational hormones, cytokine and chemokine levels as well as serum metabolites at the 23<sup>rd</sup>, 25<sup>th</sup> and 32<sup>nd</sup> week of gestation in addition to the time of delivery. Moreover, it would be possible to determine whether infections or psychological stress including severe life events induce seroconversion of islet autoantibodies or tTGA.

The validity of the methods used to detect the infections are of critical significance and both virus antibody assays and direct detection of the virus are of importance to increase the sensitivity and specificity of diagnosing virus infections. In the present investigation (Paper IV) the analyses of virus infections were limited to rRT-PCR for EV-RNA and capture ELISA for EV-IgM. Future studies should include MassTag PCR, a state-of-the-art, multiplex, PCR-based method that enables detection of bacteria, viruses, fungi and parasites in clinical samples. This method would have allowed us to simultaneously test one sample for the presence of up to 30 different agents.

Future studies of mothers in early pregnancy should also investigate mechanisms by which gestational infections, psychological stress, or both, may affect birth weight and length in children. Epidemiological studies have indicated that children developing type 1 diabetes had an increased birth weight and length, however, the mechanisms are not understood. Further studies on gestational infections are also necessary to determine their contribution to all children developing type 1 diabetes, CD, or both. This knowledge is needed before major efforts are made to subject mothers in early pregnancy to preventive measures.



# Chapter VI

## Summary and conclusion

Non-diabetic mothers during pregnancy with islet autoantibodies at delivery had significantly higher titers during early pregnancy. Seroconversion to islet autoantibodies did occur as well in non-diabetic mothers autoantibody negative in early pregnancy.

Seven out of ten cytokines (a majority of Th1 cytokines) were significantly increased in early pregnancy serum samples from mothers who later gave birth to children who developed high titers of tTGA or confirmed CD before five years of age. The increase in cytokines may be associated with CD in offspring.

Typical Th1 cytokines (IFN- $\gamma$  and IL-2) were also found to be increased in early pregnancy samples from mothers who gave birth to children developing multiple, persistent islet autoantibodies, type 1 diabetes, or both, before seven years of age.

EV-IgM in early pregnancy increased the risk for islet autoantibodies at delivery in non-diabetic mothers who carried the type 1 diabetes risk genotypes HLA-DQ 2/2 or 2/X.

Taken together, these results support the notion that gestational infections may increase the risk for type 1 diabetes or CD in the offspring.

# Sammanfattning på svenska

Typ 1-diabetes är en allvarlig, autoimmun, sjukdom och Sverige har världens näst högsta antal nya insjuknanden. Varje år diagnostiseras ca 700 individer i Sverige med sjukdomen som kan debutera i alla åldrar. Senare studier tyder dock på att barn med typ 1-diabetes blir allt yngre. Vi vet idag att ärftligheten är kopplad till en specifik gen vilket förklarar ca 30-50% av sjukdomsförekomsten. Detta tyder på att inte bara ärftlighet kan förklara sjukdomen utan någon form av miljöfaktor också krävs för att sjukdomen ska bryta ut.

Glutenintolerans är en annan autoimmun sjukdom som har samma ärftliga faktor som typ 1-diabetes, fast i denna sjukdom känner vi till antigenet, nämligen gluten - en samling proteiner som finns i vete, råg och korn.

I populationsstudien DiPiS (Diabetes Prediktion i Skåne) samlade man in blodprover från kvinnor (DiPiS mammor) i samband med förlossning. Dessa prover påvisade en säsongsberoende variation i mammor som födde barn med autoantikroppar. Dessa autoantikroppar är så kallade markörer för typ 1-diabetes. Dessutom har man visat att mammor som rapporterade infektioner tidigt under graviditeten samt under vintermånaderna, påvisade en ökad risk att föda barn med autoantikroppar. Detta skulle kunna betyda att infektioner är en faktor som påverkar förekomsten av typ 1-diabetes.

Med hjälp av en biobank vid Mikrobiologen i Malmö har vi lyckats identifiera DiPiS-mammor och hämta ut deras blodprov vilka samlades in under deras första besök hos mödravården.

Det övergripande målet med denna avhandling är att undersöka sambandet mellan miljöfaktorer under tidig graviditet och senare förekomst av autoimmunitet hos barnet. Vi testar också möjligheten att exponering för vissa virusstammar (som tillhör gruppen enterovirus) skulle kunna vara en utlösande faktor för autoimmunitet hos barnet. DiPiS-studien och mikrobiologens biobank möjliggjorde våra studier som undersöker förändringar i immunförsvaret hos mamman under tidig graviditet, som senare födde barn vilka utvecklade typ 1-diabetes eller glutenintolerans.

I den första delstudien av avhandlingen undersöker vi förekomst och utveckling av autoantikroppar kopplade till typ 1-diabetes i den gravida mamman. I detta arbete undersöktes inskrivningsprovet från Mikrobiologen i Malmö hos 242 friska mammor vid förlossning av sitt barn, för autoantikroppar mot insulin, GAD65 eller IA-2. Huvudfyndet var att de flesta mammor som födde barn med autoantikroppar hade dessa redan i inskrivningsprovet och nivån av dessa sjönk sedan under resten av

graviditeten. Detta är också vanligt med autoantikroppar för andra autoimmuna sjukdomar, där det är välkänt att de sjunker under graviditeten, till exempel ledgångsreumatism. I vår studie var det totalt 50 mammor som utvecklade autoantikroppar under graviditeten.

I det andra delarbetet undersökte vi förändringar i immunförsvaret hos mamman under tidig graviditet, samt om dessa förändringar var kopplade till glutenintolerans hos barnet senare i livet. Inskrivningsprovet från Mikrobiologen i Malmö analyserades för nio cytokiner och en chemokin, vilka alla är immunologiska markörer. Totalt visade sig sju av nio cytokiner vara förhöjda i inskrivningsprovet hos de mammor som födde ett barn som utvecklade glutenintolerans före fem års ålder.

I delarbete tre undersöktes 48 mammor som fött ett barn vilket utvecklade typ-1 diabetes före sju års ålder. Dessa mammors inskrivningsprov undersöktes tillsammans med 93 kontrollmammor för samma immunologiska markörer som i delarbete två. Huvudfynden var att cytokiner som ofta uttrycks i samband med infektioner och som aktiverar den cellulära delen av immunförsvaret återfanns i förhöjda koncentrationer hos de mammor som födde ett barn som utvecklade diabetes före sju års ålder.

I det sista delarbetet analyserades samtliga inskrivningsprover för IgM-antikroppar mot en specifik virusgrupp som kallas för enterovirus. Resultaten visar att ca 10% av mammorna visade tecken på virusexponering redan i inskrivningsprovet. Autoantikroppar återfanns oftare hos de mammor som hade den ärftliga riskfaktorn för både typ-1 diabetes och glutenintolerans. Ett viktigt fynd var att mammor som utvecklade autoantikroppar under graviditeten dvs. var negativa i inskrivningsprovet men positiva vid födseln, var i högre utsträckning positiva för enterovirus IgM-antikroppar i inskrivningsprovet än de som inte utvecklade autoantikroppar under graviditeten. Dessa resultat påvisar att en enterovirusinfektion tidig under graviditeten kan förklara att mammor med ärftlig risk för typ 1-diabetes utvecklar autoantikroppar som är karakteristiska för typ 1-diabetes.

Avhandlingen som presenteras här är den första av sitt slag då den direkt undersöker hur autoantikroppar uppstår under graviditeten och att det finns ett samband mellan inflammatoriska processer vid virusinfektioner och förekomsten av autoimmuna sjukdomar såsom glutenintolerans och typ 1-diabetes hos barnet. Detta fynd stödjer hypotesen att miljöfaktorer som påverkar mamman under tidig graviditet också påverkar det ofödda fostrets framtida hälsa.





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# References

1. Selin H. *Medicine Across Cultures: History and Practice of Medicine in Non-Western Cultures*; 2003.
2. Leopold E. Aretaeus the Cappadocian: His Contribution to Diabetes Mellitus. *Annals of Medical History* 2 1930:424-35.
3. Sakula A. Paul Langerhans (1847-1888): a centenary tribute. *J R Soc Med* 1988;81:414-5.
4. Laguesse. Sur la formation des ilots de Langerhans dans le pancreas. *Comptes Rend SocBiol* 1893;5:819-20.
5. Bliss M. *The Discovery of Insulin* University of Chicago Press; 1982.
6. Davidson. *Clinical Diabetes Mellitus: a problem-oriented approach*. 3d ed: Thieme New York; 1999.
7. Banting FG, Best CH. The internal secretion of the pancreas. 1922. *Indian J Med Res* 2007;125:251-66.
8. Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *Lancet* 2001;358:221-9.
9. Eisenbarth GS. Type I diabetes mellitus. A chronic autoimmune disease. *N Engl J Med* 1986;314:1360-8.
10. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* 1997;20:1183-97.
11. Eliasson M, Bostrom G. Chapter 5.2: major public health problems - diabetes. *Scand J Public Health Suppl* 2006;67:59-68.
12. Kilpatrick ES, Rigby AS, Atkin SL. The Diabetes Control and Complications Trial: the gift that keeps giving. *Nat Rev Endocrinol* 2009;5:537-45.
13. Bach JF. Insulin-dependent diabetes mellitus as an autoimmune disease. *Endocr Rev* 1994;15:516-42.
14. Eisenbarth GS, Linnenbach A, Jackson R, Searce R, Croce CM. Human hybridomas secreting anti-islet autoantibodies. *Nature* 1982;300:264-7.
15. Sutherland DE, Sibley R, Xu XZ, et al. Twin-to-twin pancreas transplantation: reversal and reenactment of the pathogenesis of type I diabetes. *Trans Assoc Am Physicians* 1984;97:80-7.
16. Gepts W. Pathologic anatomy of the pancreas in juvenile diabetes mellitus. *Diabetes* 1965;14:619-33.
17. Castano L, Eisenbarth GS. Type-I diabetes: a chronic autoimmune disease of human, mouse, and rat. *Annu Rev Immunol* 1990;8:647-79.
18. Pescovitz MD. Rituximab, an anti-cd20 monoclonal antibody: history and mechanism of action. *Am J Transplant* 2006;6:859-66.

19. Pescovitz MD, Greenbaum CJ, Krause-Steinrauf H, et al. Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. *N Engl J Med* 2009;361:2143-52.
20. Imagawa A, Hanafusa T, Itoh N, et al. Immunological abnormalities in islets at diagnosis paralleled further deterioration of glycaemic control in patients with recent-onset Type I (insulin-dependent) diabetes mellitus. *Diabetologia* 1999;42:574-8.
21. Effects of age, duration and treatment of insulin-dependent diabetes mellitus on residual beta-cell function: observations during eligibility testing for the Diabetes Control and Complications Trial (DCCT). The DCCT Research Group. *J Clin Endocrinol Metab* 1987;65:30-6.
22. Effect of intensive therapy on residual beta-cell function in patients with type 1 diabetes in the diabetes control and complications trial. A randomized, controlled trial. The Diabetes Control and Complications Trial Research Group. *Ann Intern Med* 1998;128:517-23.
23. Block MB, Rosenfield RL, Mako ME, Steiner DF, Rubenstein AH. Sequential changes in beta-cell function in insulin-treated diabetic patients assessed by C-peptide immunoreactivity. *N Engl J Med* 1973;288:1144-8.
24. Green A, Patterson CC. Trends in the incidence of childhood-onset diabetes in Europe 1989-1998. *Diabetologia* 2001;44 Suppl 3:B3-8.
25. Onkamo P, Vaananen S, Karvonen M, Tuomilehto J. Worldwide increase in incidence of Type I diabetes--the analysis of the data on published incidence trends. *Diabetologia* 1999;42:1395-403.
26. Karvonen M, Pitkaniemi J, Tuomilehto J. The onset age of type 1 diabetes in Finnish children has become younger. The Finnish Childhood Diabetes Registry Group. *Diabetes Care* 1999;22:1066-70.
27. Charkaluk ML, Czernichow P, Levy-Marchal C. Incidence data of childhood-onset type I diabetes in France during 1988-1997: the case for a shift toward younger age at onset. *Pediatr Res* 2002;52:859-62.
28. Variation and trends in incidence of childhood diabetes in Europe. EURODIAB ACE Study Group. *Lancet* 2000;355:873-6.
29. ADA. Geographic patterns of childhood insulin-dependent diabetes mellitus. Diabetes Epidemiology Research International Group. *Diabetes* 1988;37:1113-9.
30. Rewers M, LaPorte RE, King H, Tuomilehto J. Trends in the prevalence and incidence of diabetes: insulin-dependent diabetes mellitus in childhood. *World Health Stat Q* 1988;41:179-89.
31. Pundziute-Lycka A, Dahlquist G, Nystrom L, et al. The incidence of Type I diabetes has not increased but shifted to a younger age at diagnosis in the 0-34 years group in Sweden 1983-1998. *Diabetologia* 2002;45:783-91.
32. Sabbah E, Savola K, Ebeling T, et al. Genetic, autoimmune, and clinical characteristics of childhood- and adult-onset type 1 diabetes. *Diabetes Care* 2000;23:1326-32.
33. Karvonen M, Pitkaniemi M, Pitkaniemi J, Kohtamaki K, Tajima N, Tuomilehto J. Sex difference in the incidence of insulin-dependent diabetes mellitus:

an analysis of the recent epidemiological data. World Health Organization DIAMOND Project Group. *Diabetes Metab Rev* 1997;13:275-91.

34. Williams AJ, Bingley PJ, Moore WP, Gale EA. Islet autoantibodies, nationality and gender: a multinational screening study in first-degree relatives of patients with Type I diabetes. *Diabetologia* 2002;45:217-23.

35. Kondrashova A, Reunanen A, Romanov A, et al. A six-fold gradient in the incidence of type 1 diabetes at the eastern border of Finland. *Ann Med* 2005;37:67-72.

36. Menser MA, Dods L, Harley JD. A twenty-five-year follow-up of congenital rubella. *Lancet* 1967;2:1347-50.

37. Knip M, Veijola R, Virtanen SM, Hyoty H, Vaarala O, Akerblom HK. Environmental triggers and determinants of type 1 diabetes. *Diabetes* 2005;54 Suppl 2:S125-36.

38. Akerblom HK, Vaarala O, Hyoty H, Ilonen J, Knip M. Environmental factors in the etiology of type 1 diabetes. *Am J Med Genet* 2002;115:18-29.

39. Concannon P, Erlich HA, Julier C, et al. Type 1 diabetes: evidence for susceptibility loci from four genome-wide linkage scans in 1,435 multiplex families. *Diabetes* 2005;54:2995-3001.

40. Owerbach D, Lernmark A, Platz P, et al. HLA-D region beta-chain DNA endonuclease fragments differ between HLA-DR identical healthy and insulin-dependent diabetic individuals. *Nature* 1983;303:815-7.

41. Kockum I, Sanjeevi CB, Eastman S, Landin-Olsson M, Dahlquist G, Lernmark A. Complex interaction between HLA DR and DQ in conferring risk for childhood type 1 diabetes. *Eur J Immunogenet* 1999;26:361-72.

42. Lucassen AM, Julier C, Beressi JP, et al. Susceptibility to insulin dependent diabetes mellitus maps to a 4.1 kb segment of DNA spanning the insulin gene and associated VNTR. *Nat Genet* 1993;4:305-10.

43. Bell GI, Horita S, Karam JH. A polymorphic locus near the human insulin gene is associated with insulin-dependent diabetes mellitus. *Diabetes* 1984;33:176-83.

44. Marron MP, Raffel LJ, Garchon HJ, et al. Insulin-dependent diabetes mellitus (IDDM) is associated with CTLA4 polymorphisms in multiple ethnic groups. *Hum Mol Genet* 1997;6:1275-82.

45. Pugliese A. Genetics of type 1 diabetes. *Endocrinol Metab Clin North Am* 2004;33:1-16, vii.

46. Singal DP, Blajchman MA. Histocompatibility (HL-A) antigens, lymphocytotoxic antibodies and tissue antibodies in patients with diabetes mellitus. *Diabetes* 1973;22:429-32.

47. Nerup J, Platz P, Andersen OO, et al. HL-A antigens and diabetes mellitus. *Lancet* 1974;2:864-6.

48. Bhatia E, Mehra NK, Malaviya AN, Ahuja MM. HLA and autoimmunity in North Indian type I (insulin-dependent) diabetic multiplex families. *Horm Metab Res* 1986;18:331-4.

49. Graham J, Hagopian WA, Kockum I, et al. Genetic effects on age-dependent onset and islet cell autoantibody markers in type 1 diabetes. *Diabetes* 2002;51:1346-55.
50. Komulainen J, Kulmala P, Savola K, et al. Clinical, autoimmune, and genetic characteristics of very young children with type 1 diabetes. Childhood Diabetes in Finland (DiMe) Study Group. *Diabetes Care* 1999;22:1950-5.
51. Andersson C, Larsson K, Vaziri-Sani F, et al. The three ZNT8 autoantibody variants together improve the diagnostic sensitivity of childhood and adolescent type 1 diabetes. *Autoimmunity*.
52. Sanjeevi CB, Hjelmstrom P, Hallmans G, et al. Different HLA-DR-DQ haplotypes are associated with cervical intraepithelial neoplasia among human papillomavirus type-16 seropositive and seronegative Swedish women. *Int J Cancer* 1996;68:409-14.
53. Nepom BS, Nepom GT, Mickelson E, Schaller JG, Antonelli P, Hansen JA. Specific HLA-DR4-associated histocompatibility molecules characterize patients with seropositive juvenile rheumatoid arthritis. *J Clin Invest* 1984;74:287-91.
54. Michelsen B, Lernmark A. Molecular cloning of a polymorphic DNA endonuclease fragment associates insulin-dependent diabetes mellitus with HLA-DQ. *J Clin Invest* 1987;79:1144-52.
55. Todd JA, Mijovic C, Fletcher J, Jenkins D, Bradwell AR, Barnett AH. Identification of susceptibility loci for insulin-dependent diabetes mellitus by trans-racial gene mapping. *Nature* 1989;338:587-9.
56. Barbosa J, Chern MM, Anderson VE, et al. Linkage analysis between the major histocompatibility system and insulin-dependent diabetes in families with patients in two consecutive generations. *J Clin Invest* 1980;65:592-601.
57. Scherthaner G, Banatvala JE, Scherbaum W, et al. Coxsackie-B-virus-specific IgM responses, complement-fixing islet-cell antibodies, HLA DR antigens, and C-peptide secretion in insulin-dependent diabetes mellitus. *Lancet* 1985;2:630-2.
58. Sadeharju K, Knip M, Hiltunen M, Akerblom HK, Hyoty H. The HLA-DR phenotype modulates the humoral immune response to enterovirus antigens. *Diabetologia* 2003;46:1100-5.
59. Bruserud O, Jervell J, Thorsby E. HLA-DR3 and -DR4 control T-lymphocyte responses to mumps and Coxsackie B4 virus: studies on patients with type 1 (insulin-dependent) diabetes and healthy subjects. *Diabetologia* 1985;28:420-6.
60. Knip M, Siljander H. Autoimmune mechanisms in type 1 diabetes. *Autoimmun Rev* 2008;7:550-7.
61. Foulis AK, Stewart JA. The pancreas in recent-onset type 1 (insulin-dependent) diabetes mellitus: insulin content of islets, insulinitis and associated changes in the exocrine acinar tissue. *Diabetologia* 1984;26:456-61.
62. Borg H, Arnqvist HJ, Bjork E, et al. Evaluation of the new ADA and WHO criteria for classification of diabetes mellitus in young adult people (15-34 yrs) in the Diabetes Incidence Study in Sweden (DISS). *Diabetologia* 2003;46:173-81.
63. Wenzlau JM, Juhl K, Yu L, et al. The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. *Proc Natl Acad Sci U S A* 2007;104:17040-5.



64. Pihoker C, Gilliam LK, Hampe CS, Lernmark A. Autoantibodies in diabetes. *Diabetes* 2005;54 Suppl 2:S52-61.
65. Eisenbarth GS. Prediction of type 1 diabetes: the natural history of the prediabetic period. *Adv Exp Med Biol* 2004;552:268-90.
66. Atkinson MA, Maclaren NK. The pathogenesis of insulin-dependent diabetes mellitus. *N Engl J Med* 1994;331:1428-36.
67. Gale EA, Bingley PJ, Emmett CL, Collier T. European Nicotinamide Diabetes Intervention Trial (ENDIT): a randomised controlled trial of intervention before the onset of type 1 diabetes. *Lancet* 2004;363:925-31.
68. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* 1998;15:539-53.
69. ADA. Report of the expert committee on the diagnosis and classification of diabetes mellitus. *Diabetes Care* 2003;26 Suppl 1:S5-20.
70. Borg H, Gottsater A, Fernlund P, Sundkvist G. A 12-year prospective study of the relationship between islet antibodies and beta-cell function at and after the diagnosis in patients with adult-onset diabetes. *Diabetes* 2002;51:1754-62.
71. Martin S, Wolf-Eichbaum D, Duinkerken G, et al. Development of type 1 diabetes despite severe hereditary B-lymphocyte deficiency. *N Engl J Med* 2001;345:1036-40.
72. Petersen JS, Dyrberg T, Karlsen AE, et al. Glutamic acid decarboxylase (GAD65) autoantibodies in prediction of beta-cell function and remission in recent-onset IDDM after cyclosporin treatment. The Canadian-European Randomized Control Trial Group. *Diabetes* 1994;43:1291-6.
73. Baekkeskov S, Aanstoot HJ, Christgau S, et al. Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature* 1990;347:151-6.
74. Martin DL, Rimvall K. Regulation of gamma-aminobutyric acid synthesis in the brain. *J Neurochem* 1993;60:395-407.
75. Reetz A, Solimena M, Matteoli M, Folli F, Takei K, De Camilli P. GABA and pancreatic beta-cells: colocalization of glutamic acid decarboxylase (GAD) and GABA with synaptic-like microvesicles suggests their role in GABA storage and secretion. *EMBO J* 1991;10:1275-84.
76. Baekkeskov S, Nielsen JH, Marner B, Bilde T, Ludvigsson J, Lernmark A. Autoantibodies in newly diagnosed diabetic children immunoprecipitate human pancreatic islet cell proteins. *Nature* 1982;298:167-9.
77. Karlsen AE, Hagopian WA, Grubin CE, et al. Cloning and primary structure of a human islet isoform of glutamic acid decarboxylase from chromosome 10. *Proc Natl Acad Sci U S A* 1991;88:8337-41.
78. Bu DF, Tobin AJ. The exon-intron organization of the genes (GAD1 and GAD2) encoding two human glutamate decarboxylases (GAD67 and GAD65) suggests that they derive from a common ancestral GAD. *Genomics* 1994;21:222-8.
79. Erlander MG, Tillakaratne NJ, Feldblum S, Patel N, Tobin AJ. Two genes encode distinct glutamate decarboxylases. *Neuron* 1991;7:91-100.

80. Bu DF, Erlander MG, Hitz BC, et al. Two human glutamate decarboxylases, 65-kDa GAD and 67-kDa GAD, are each encoded by a single gene. *Proc Natl Acad Sci U S A* 1992;89:2115-9.
81. Lernmark A. Glutamic acid decarboxylase--gene to antigen to disease. *J Intern Med* 1996;240:259-77.
82. Solimena M, Folli F, Aparisi R, Pozza G, De Camilli P. Autoantibodies to GABA-ergic neurons and pancreatic beta cells in stiff-man syndrome. *N Engl J Med* 1990;322:1555-60.
83. Bjork E, Velloso LA, Kampe O, Karlsson FA. GAD autoantibodies in IDDM, stiff-man syndrome, and autoimmune polyendocrine syndrome type I recognize different epitopes. *Diabetes* 1994;43:161-5.
84. Li L, Hagopian WA, Brashear HR, Daniels T, Lernmark A. Identification of autoantibody epitopes of glutamic acid decarboxylase in stiff-man syndrome patients. *J Immunol* 1994;152:930-4.
85. Daw K, Ujihara N, Atkinson M, Powers AC. Glutamic acid decarboxylase autoantibodies in stiff-man syndrome and insulin-dependent diabetes mellitus exhibit similarities and differences in epitope recognition. *J Immunol* 1996;156:818-25.
86. Vives-Pi M, Somoza N, Vargas F, et al. Expression of glutamic acid decarboxylase (GAD) in the alpha, beta and delta cells of normal and diabetic pancreas: implications for the pathogenesis of type I diabetes. *Clin Exp Immunol* 1993;92:391-6.
87. Hagopian WA, Sanjeevi CB, Kockum I, et al. Glutamate decarboxylase-, insulin-, and islet cell-antibodies and HLA typing to detect diabetes in a general population-based study of Swedish children. *J Clin Invest* 1995;95:1505-11.
88. Genovese S, Bonfanti R, Bazzigaluppi E, et al. Association of IA-2 autoantibodies with HLA DR4 phenotypes in IDDM. *Diabetologia* 1996;39:1223-6.
89. Falorni A, Ortqvist E, Persson B, Lernmark A. Radioimmunoassays for glutamic acid decarboxylase (GAD65) and GAD65 autoantibodies using 35S or 3H recombinant human ligands. *J Immunol Methods* 1995;186:89-99.
90. Vandewalle CL, Falorni A, Svanholm S, Lernmark A, Pipeleers DG, Gorus FK. High diagnostic sensitivity of glutamate decarboxylase autoantibodies in insulin-dependent diabetes mellitus with clinical onset between age 20 and 40 years. The Belgian Diabetes Registry. *J Clin Endocrinol Metab* 1995;80:846-51.
91. Turner R, Stratton I, Horton V, et al. UKPDS 25: autoantibodies to islet-cell cytoplasm and glutamic acid decarboxylase for prediction of insulin requirement in type 2 diabetes. UK Prospective Diabetes Study Group. *Lancet* 1997;350:1288-93.
92. Bingley PJ, Christie MR, Bonifacio E, et al. Combined analysis of autoantibodies improves prediction of IDDM in islet cell antibody-positive relatives. *Diabetes* 1994;43:1304-10.
93. Christie MR, Genovese S, Cassidy D, et al. Antibodies to islet 37k antigen, but not to glutamate decarboxylase, discriminate rapid progression to IDDM in endocrine autoimmunity. *Diabetes* 1994;43:1254-9.

94. Aguilar-Diosdado M, Parkinson D, Corbett JA, et al. Potential autoantigens in IDDM. Expression of carboxypeptidase-H and insulin but not glutamate decarboxylase on the beta-cell surface. *Diabetes* 1994;43:418-25.
95. Tsirogianni A, Pipi E, Soufleros K. Specificity of islet cell autoantibodies and coexistence with other organ specific autoantibodies in type 1 diabetes mellitus. *Autoimmun Rev* 2009;8:687-91.
96. Christie MR, Tun RY, Lo SS, et al. Antibodies to GAD and tryptic fragments of islet 64K antigen as distinct markers for development of IDDM. Studies with identical twins. *Diabetes* 1992;41:782-7.
97. Christie DJ, Sauro SC, Fairbanks KD, Kay NE. Detection of clonal platelet antibodies in immunologically-mediated thrombocytopenias: association with circulating clonal/oligoclonal B cells. *Br J Haematol* 1993;85:277-84.
98. Lan MS, Wasserfall C, Maclaren NK, Notkins AL. IA-2, a transmembrane protein of the protein tyrosine phosphatase family, is a major autoantigen in insulin-dependent diabetes mellitus. *Proc Natl Acad Sci U S A* 1996;93:6367-70.
99. Bonifacio E, Lampasona V, Genovese S, Ferrari M, Bosi E. Identification of protein tyrosine phosphatase-like IA2 (islet cell antigen 512) as the insulin-dependent diabetes-related 37/40K autoantigen and a target of islet-cell antibodies. *J Immunol* 1995;155:5419-26.
100. Solimena M, Dirx R, Jr., Hermel JM, et al. ICA 512, an autoantigen of type I diabetes, is an intrinsic membrane protein of neurosecretory granules. *EMBO J* 1996;15:2102-14.
101. Lampasona V, Bearzatto M, Genovese S, Bosi E, Ferrari M, Bonifacio E. Autoantibodies in insulin-dependent diabetes recognize distinct cytoplasmic domains of the protein tyrosine phosphatase-like IA-2 autoantigen. *J Immunol* 1996;157:2707-11.
102. Graham J, Kockum I, Sanjeevi CB, et al. Negative association between type 1 diabetes and HLA DQB1\*0602-DQA1\*0102 is attenuated with age at onset. Swedish Childhood Diabetes Study Group. *Eur J Immunogenet* 1999;26:117-27.
103. Savola K, Bonifacio E, Sabbah E, et al. IA-2 antibodies--a sensitive marker of IDDM with clinical onset in childhood and adolescence. Childhood Diabetes in Finland Study Group. *Diabetologia* 1998;41:424-9.
104. Hawa MI, Fava D, Medici F, et al. Antibodies to IA-2 and GAD65 in type 1 and type 2 diabetes: isotype restriction and polyclonality. *Diabetes Care* 2000;23:228-33.
105. Bingley PJ, Bonifacio E, Mueller PW. Diabetes Antibody Standardization Program: first assay proficiency evaluation. *Diabetes* 2003;52:1128-36.
106. Delli AJ, Lindblad B, Carlsson A, et al. Type 1 diabetes patients born to immigrants to Sweden increase their native diabetes risk and differ from Swedish patients in HLA types and islet autoantibodies. *Pediatr Diabetes*.
107. Palmer JP. Insulin autoantibodies: their role in the pathogenesis of IDDM. *Diabetes Metab Rev* 1987;3:1005-15.

108. Barker JM, Barriga KJ, Yu L, et al. Prediction of autoantibody positivity and progression to type 1 diabetes: Diabetes Autoimmunity Study in the Young (DAISY). *J Clin Endocrinol Metab* 2004;89:3896-902.
109. Yu L. Humoral Autoimmunity Type 1 diabetes: molecular, cellular and clinical immunology. Online edition ed; 2007.
110. Gorus FK, Vandewalle CL, Dorchy H, Van Crombrugge P, Schuit FC, Pipeleers DG. Influence of age on the associations among insulin autoantibodies, islet cell antibodies, and HLA DAQ1\*0301-DQB1\*0302 in siblings of patients with type 1 (insulin-dependent) diabetes mellitus. Belgian Diabetes Registry. *J Clin Endocrinol Metab* 1994;78:1172-8.
111. Williams AJ, Norcross AJ, Dix RJ, Gillespie KM, Gale EA, Bingley PJ. The prevalence of insulin autoantibodies at the onset of Type 1 diabetes is higher in males than females during adolescence. *Diabetologia* 2003;46:1354-6.
112. Pugliese A, Bugawan T, Moromisato R, et al. Two subsets of HLA-DQA1 alleles mark phenotypic variation in levels of insulin autoantibodies in first degree relatives at risk for insulin-dependent diabetes. *J Clin Invest* 1994;93:2447-52.
113. Achenbach P, Koczwara K, Knopff A, Naserke H, Ziegler AG, Bonifacio E. Mature high-affinity immune responses to (pro)insulin anticipate the autoimmune cascade that leads to type 1 diabetes. *J Clin Invest* 2004;114:589-97.
114. Ziegler AG, Hummel M, Schenker M, Bonifacio E. Autoantibody appearance and risk for development of childhood diabetes in offspring of parents with type 1 diabetes: the 2-year analysis of the German BABYDIAB Study. *Diabetes* 1999;48:460-8.
115. Rewers M, Norris JM, Eisenbarth GS, et al. Beta-cell autoantibodies in infants and toddlers without IDDM relatives: diabetes autoimmunity study in the young (DAISY). *J Autoimmun* 1996;9:405-10.
116. Greener M. PANDA identifies babies at risk of developing type 1 diabetes. *Mol Med Today* 2000;6:3.
117. Verge CF, Gianani R, Kawasaki E, et al. Number of autoantibodies (against insulin, GAD or ICA512/IA2) rather than particular autoantibody specificities determines risk of type I diabetes. *J Autoimmun* 1996;9:379-83.
118. Verge CF, Gianani R, Kawasaki E, et al. Prediction of type I diabetes in first-degree relatives using a combination of insulin, GAD, and ICA512bdc/IA-2 autoantibodies. *Diabetes* 1996;45:926-33.
119. Yu L, Rewers M, Gianani R, et al. Antiislet autoantibodies usually develop sequentially rather than simultaneously. *J Clin Endocrinol Metab* 1996;81:4264-7.
120. Christie MR, Hollands JA, Brown TJ, Michelsen BK, Delovitch TL. Detection of pancreatic islet 64,000 M(r) autoantigens in insulin-dependent diabetes distinct from glutamate decarboxylase. *J Clin Invest* 1993;92:240-8.
121. Ziegler AG, Hillebrand B, Rabl W, et al. On the appearance of islet associated autoimmunity in offspring of diabetic mothers: a prospective study from birth. *Diabetologia* 1993;36:402-8.
122. Hayward AR, ed. Development of immunity mechanism Blackwell scientific publications Oxford; 1981.

123. Stanley HM, Norris JM, Barriga K, et al. Is presence of islet autoantibodies at birth associated with development of persistent islet autoimmunity? The Diabetes Autoimmunity Study in the Young (DAISY). *Diabetes Care* 2004;27:497-502.
124. Lynch KF, Lernmark B, Merlo J, Cilio CM, Ivarsson SA, Lernmark A. Cord blood islet autoantibodies and seasonal association with the type 1 diabetes high-risk genotype. *J Perinatol* 2008;28:211-7.
125. Ivarsson SA, Ackefors M, Carlsson A, et al. Glutamate decarboxylase antibodies in non-diabetic pregnancy precedes insulin-dependent diabetes in the mother but not necessarily in the offspring. *Autoimmunity* 1997;26:261-9.
126. Lindberg B, Ivarsson SA, Landin-Olsson M, Sundkvist G, Svanberg L, Lernmark A. Islet autoantibodies in cord blood from children who developed type 1 (insulin-dependent) diabetes mellitus before 15 years of age. *Diabetologia* 1999;42:181-7.
127. Elfving AM, Lindberg BA, Nystrom L, Sundkvist G, Lernmark A, Ivarsson SA. Islet autoantibodies in cord blood from patients who developed type 1 diabetes mellitus at 15-30 years of age. *Autoimmunity* 2003;36:227-31.
128. Koczwara K, Bonifacio E, Ziegler AG. Transmission of maternal islet antibodies and risk of autoimmune diabetes in offspring of mothers with type 1 diabetes. *Diabetes* 2004;53:1-4.
129. Eising S, Nilsson A, Carstensen B, et al. Danish children born with glutamic acid decarboxylase-65 and islet antigen-2 autoantibodies at birth had an increased risk to develop type 1 diabetes. *Eur J Endocrinol*;164:247-52.
130. Dahlquist G. Etiological aspects of insulin-dependent diabetes mellitus: an epidemiological perspective. *Autoimmunity* 1993;15:61-5.
131. Padaiga Z, Tuomilehto J, Karvonen M, et al. Seasonal variation in the incidence of Type 1 diabetes mellitus during 1983 to 1992 in the countries around the Baltic Sea. *Diabet Med* 1999;16:736-43.
132. Kondrashova A, Viskari H, Kulmala P, et al. Signs of beta-cell autoimmunity in nondiabetic schoolchildren: a comparison between Russian Karelia with a low incidence of type 1 diabetes and Finland with a high incidence rate. *Diabetes Care* 2007;30:95-100.
133. Menser MA, Forrest JM, Honeyman MC, Burgess JA. Letter: Diabetes, HL-A antigens, and congenital rubella. *Lancet* 1974;2:1508-9.
134. Ginsberg-Fellner F, Witt ME, Yagihashi S, et al. Congenital rubella syndrome as a model for type 1 (insulin-dependent) diabetes mellitus: increased prevalence of islet cell surface antibodies. *Diabetologia* 1984;27 Suppl:87-9.
135. Menser MA, Forrest JM, Bransby RD. Rubella infection and diabetes mellitus. *Lancet* 1978;1:57-60.
136. McIntosh ED, Menser MA. A fifty-year follow-up of congenital rubella. *Lancet* 1992;340:414-5.
137. Ou D, Mitchell LA, Metzger DL, Gillam S, Tingle AJ. Cross-reactive rubella virus and glutamic acid decarboxylase (65 and 67) protein determinants recognised by T cells of patients with type 1 diabetes mellitus. *Diabetologia* 2000;43:750-62.

138. Cavallo MG, Baroni MG, Toto A, et al. Viral infection induces cytokine release by beta islet cells. *Immunology* 1992;75:664-8.
139. DeStefano F, Mullooly JP, Okoro CA, et al. Childhood vaccinations, vaccination timing, and risk of type 1 diabetes mellitus. *Pediatrics* 2001;108:E112.
140. Hiltunen M, Lonnrot M, Hyoty H. Immunisation and type 1 diabetes mellitus: is there a link? *Drug Saf* 1999;20:207-12.
141. Honeyman MC, Coulson BS, Stone NL, et al. Association between rotavirus infection and pancreatic islet autoimmunity in children at risk of developing type 1 diabetes. *Diabetes* 2000;49:1319-24.
142. Blomqvist M, Juhela S, Erkkila S, et al. Rotavirus infections and development of diabetes-associated autoantibodies during the first 2 years of life. *Clin Exp Immunol* 2002;128:511-5.
143. Infections and vaccinations as risk factors for childhood type I (insulin-dependent) diabetes mellitus: a multicentre case-control investigation. EURODIAB Substudy 2 Study Group. *Diabetologia* 2000;43:47-53.
144. Stene LC, Barriga K, Norris JM, et al. Symptoms of common maternal infections in pregnancy and risk of islet autoimmunity in early childhood. *Diabetes Care* 2003;26:3136-41.
145. Helmke K, Otten A, Willems W. Islet cell antibodies in children with mumps infection. *Lancet* 1980;2:211-2.
146. Hyoty H, Leinikki P, Reunanen A, et al. Mumps infections in the etiology of type 1 (insulin-dependent) diabetes. *Diabetes Res* 1988;9:111-6.
147. Viskari H, Paronen J, Keskinen P, et al. Humoral beta-cell autoimmunity is rare in patients with the congenital rubella syndrome. *Clin Exp Immunol* 2003;133:378-83.
148. Coulson BS, Witterick PD, Tan Y, et al. Growth of rotaviruses in primary pancreatic cells. *J Virol* 2002;76:9537-44.
149. Ward KP, Galloway WH, Auchterlonie IA. Congenital cytomegalovirus infection and diabetes. *Lancet* 1979;1:497.
150. Ivarsson SA, Lindberg B, Nilsson KO, Ahlfors K, Svanberg L. The prevalence of type 1 diabetes mellitus at follow-up of Swedish infants congenitally infected with cytomegalovirus. *Diabet Med* 1993;10:521-3.
151. Hao W, Serreze DV, McCulloch DK, Neifing JL, Palmer JP. Insulin (auto)antibodies from human IDDM cross-react with retroviral antigen p73. *J Autoimmun* 1993;6:787-98.
152. Conrad B, Weissmahr RN, Boni J, Arcari R, Schupbach J, Mach B. A human endogenous retroviral superantigen as candidate autoimmune gene in type I diabetes. *Cell* 1997;90:303-13.
153. Badenhoop K, Donner H, Neumann J, et al. IDDM patients neither show humoral reactivities against endogenous retroviral envelope protein nor do they differ in retroviral mRNA expression from healthy relatives or normal individuals. *Diabetes* 1999;48:215-8.
154. Onitilo AA, Shaw GR. Parvovirus B19 infection in an isolated pancreas transplant recipient. *Transplant Proc* 2005;37:4433-5.

155. Munakata Y, Kodera T, Saito T, Sasaki T. Rheumatoid arthritis, type 1 diabetes, and Graves' disease after acute parvovirus B19 infection. *Lancet* 2005;366:780.
156. O'Brayan TA, Beck MJ, Demers LM, Naides SJ. Human parvovirus B19 infection in children with new onset Type 1 diabetes mellitus. *Diabet Med* 2005;22:1778-9.
157. Surcel HM, Ilonen J, Kaar ML, Hyoty H, Leinikki P. Infection by multiple viruses and lymphocyte abnormalities at the diagnosis of diabetes. *Acta Paediatr Scand* 1988;77:471-4.
158. Horn GT, Bugawan TL, Long CM, Erlich HA. Allelic sequence variation of the HLA-DQ loci: relationship to serology and to insulin-dependent diabetes susceptibility. *Proc Natl Acad Sci U S A* 1988;85:6012-6.
159. Sairenji T, Daibata M, Sorli CH, et al. Relating homology between the Epstein-Barr virus BOLF1 molecule and HLA-DQw8 beta chain to recent onset type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 1991;34:33-9.
160. Melnick JL. My role in the discovery and classification of the enteroviruses. *Annu Rev Microbiol* 1996;50:1-24.
161. Oberste MS, Maher K, Kilpatrick DR, Flemister MR, Brown BA, Pallansch MA. Typing of human enteroviruses by partial sequencing of VP1. *J Clin Microbiol* 1999;37:1288-93.
162. Yoon JW. The role of viruses and environmental factors in the induction of diabetes. *Curr Top Microbiol Immunol* 1990;164:95-123.
163. Kaplan MH, Klein SW, McPhee J, Harper RG. Group B coxsackievirus infections in infants younger than three months of age: a serious childhood illness. *Rev Infect Dis* 1983;5:1019-32.
164. Jenista JA, Powell KR, Menegus MA. Epidemiology of neonatal enterovirus infection. *J Pediatr* 1984;104:685-90.
165. Pallansch MA, Raymond P. Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. . 4th ed: Lippincott-Williams & Wilkins; 2001.
166. Richer MJ, Horwitz MS. Coxsackievirus infection as an environmental factor in the etiology of type 1 diabetes. *Autoimmun Rev* 2009;8:611-5.
167. Gamble DR, Taylor KW, Cumming H. Coxsackie viruses and diabetes mellitus. *Br Med J* 1973;4:260-2.
168. Craighead JE, McLane MF. Diabetes mellitus: induction in mice by encephalomyocarditis virus. *Science* 1968;162:913-4.
169. Yin H, Berg AK, Tuvemo T, Frisk G. Enterovirus RNA is found in peripheral blood mononuclear cells in a majority of type 1 diabetic children at onset. *Diabetes* 2002;51:1964-71.
170. Frisk G, Nilsson E, Tuvemo T, Friman G, Diderholm H. The possible role of Coxsackie A and echo viruses in the pathogenesis of type I diabetes mellitus studied by IgM analysis. *J Infect* 1992;24:13-22.
171. Stene LC, Oikarinen S, Hyoty H, et al. Enterovirus Infection and Progression from Islet Autoimmunity to Type 1 Diabetes: The Diabetes and Autoimmunity Study in the Young (DAISY). *Diabetes*.

172. Lonnrot M, Korpela K, Knip M, et al. Enterovirus infection as a risk factor for beta-cell autoimmunity in a prospectively observed birth cohort: the Finnish Diabetes Prediction and Prevention Study. *Diabetes* 2000;49:1314-8.
173. Hiltunen M, Hyoty H, Knip M, et al. Islet cell antibody seroconversion in children is temporally associated with enterovirus infections. Childhood Diabetes in Finland (DiMe) Study Group. *J Infect Dis* 1997;175:554-60.
174. Hyoty H, Hiltunen M, Knip M, et al. A prospective study of the role of coxsackie B and other enterovirus infections in the pathogenesis of IDDM. Childhood Diabetes in Finland (DiMe) Study Group. *Diabetes* 1995;44:652-7.
175. Dahlquist G, Frisk G, Ivarsson SA, Svanberg L, Forsgren M, Diderholm H. Indications that maternal coxsackie B virus infection during pregnancy is a risk factor for childhood-onset IDDM. *Diabetologia* 1995;38:1371-3.
176. Modlin JF, Bowman M. Perinatal transmission of coxsackievirus B3 in mice. *J Infect Dis* 1987;156:21-5.
177. Euscher E, Davis J, Holzman I, Nuovo GJ. Coxsackie virus infection of the placenta associated with neurodevelopmental delays in the newborn. *Obstet Gynecol* 2001;98:1019-26.
178. Bates HR, Jr. Coxsackie virus B3 calcific pancarditis and hydrops fetalis. *Am J Obstet Gynecol* 1970;106:629-30.
179. Sauerbrei A, Gluck B, Jung K, Bittrich H, Wutzler P. Congenital skin lesions caused by intrauterine infection with coxsackievirus B3. *Infection* 2000;28:326-8.
180. Smalling TW, Sefers SE, Li H, Tang YW. Molecular approaches to detecting herpes simplex virus and enteroviruses in the central nervous system. *J Clin Microbiol* 2002;40:2317-22.
181. Ouellet A, Sherlock R, Teye B, Fung KF. Antenatal diagnosis of intrauterine infection with coxsackievirus B3 associated with live birth. *Infect Dis Obstet Gynecol* 2004;12:23-6.
182. Dahlquist GG, Ivarsson S, Lindberg B, Forsgren M. Maternal enteroviral infection during pregnancy as a risk factor for childhood IDDM. A population-based case-control study. *Diabetes* 1995;44:408-13.
183. Dahlquist GG, Boman JE, Juto P. Enteroviral RNA and IgM antibodies in early pregnancy and risk for childhood-onset IDDM in offspring. *Diabetes Care* 1999;22:364-5.
184. Elfving M, Svensson J, Oikarinen S, et al. Maternal enterovirus infection during pregnancy as a risk factor in offspring diagnosed with type 1 diabetes between 15 and 30 years of age. *Exp Diabetes Res* 2008;2008:271958.
185. Fuchtenbusch M, Irnstetter A, Jager G, Ziegler AG. No evidence for an association of coxsackie virus infections during pregnancy and early childhood with development of islet autoantibodies in offspring of mothers or fathers with type 1 diabetes. *J Autoimmun* 2001;17:333-40.
186. Viskari HR, Roivainen M, Reunanen A, et al. Maternal first-trimester enterovirus infection and future risk of type 1 diabetes in the exposed fetus. *Diabetes* 2002;51:2568-71.



187. Salminen K, Sadeharju K, Lonrot M, et al. Enterovirus infections are associated with the induction of beta-cell autoimmunity in a prospective birth cohort study. *J Med Virol* 2003;69:91-8.
188. Lonrot M, Salminen K, Knip M, et al. Enterovirus RNA in serum is a risk factor for beta-cell autoimmunity and clinical type 1 diabetes: a prospective study. Childhood Diabetes in Finland (DiMe) Study Group. *J Med Virol* 2000;61:214-20.
189. Asplin CM, Cooney MK, Crossley JR, Dornan TL, Raghu P, Palmer JP. Coxsackie B4 infection and islet cell antibodies three years before overt diabetes. *J Pediatr* 1982;101:398-400.
190. Nigro G, Pacella ME, Patane E, Midulla M. Multi-system coxsackievirus B-6 infection with findings suggestive of diabetes mellitus. *Eur J Pediatr* 1986;145:557-9.
191. Sadeharju K, Hamalainen AM, Knip M, et al. Enterovirus infections as a risk factor for type 1 diabetes: virus analyses in a dietary intervention trial. *Clin Exp Immunol* 2003;132:271-7.
192. Graves PM, Rotbart HA, Nix WA, et al. Prospective study of enteroviral infections and development of beta-cell autoimmunity. Diabetes autoimmunity study in the young (DAISY). *Diabetes Res Clin Pract* 2003;59:51-61.
193. Tapia G, Cinek O, Rasmussen T, et al. Human Enterovirus RNA in Monthly Fecal Samples and Islet Autoimmunity in Norwegian Children With High Genetic Risk for Type 1 Diabetes: The MIDIA study. *Diabetes Care*;34:151-5.
194. Tapia G, Cinek O, Rasmussen T, Grinde B, Stene LC, Ronningen KS. Longitudinal study of parechovirus infection in infancy and risk of repeated positivity for multiple islet autoantibodies: the MIDIA study. *Pediatr Diabetes*;12:58-62.
195. Lonrot M, Knip M, Roivainen M, Koskela P, Akerblom HK, Hyoty H. Onset of type 1 diabetes mellitus in infancy after enterovirus infections. *Diabet Med* 1998;15:431-4.
196. Williams CH, Oikarinen S, Tauriainen S, Salminen K, Hyoty H, Stanway G. Molecular analysis of an echovirus 3 strain isolated from an individual concurrently with appearance of islet cell and IA-2 autoantibodies. *J Clin Microbiol* 2006;44:441-8.
197. Gamble DR, Kinsley ML, FitzGerald MG, Bolton R, Taylor KW. Viral antibodies in diabetes mellitus. *Br Med J* 1969;3:627-30.
198. Yoon JW, Austin M, Onodera T, Notkins AL. Isolation of a virus from the pancreas of a child with diabetic ketoacidosis. *N Engl J Med* 1979;300:1173-9.
199. Dotta F, Censini S, van Halteren AG, et al. Coxsackie B4 virus infection of beta cells and natural killer cell insulinitis in recent-onset type 1 diabetic patients. *Proc Natl Acad Sci U S A* 2007;104:5115-20.
200. Ylipaasto P, Klingel K, Lindberg AM, et al. Enterovirus infection in human pancreatic islet cells, islet tropism in vivo and receptor involvement in cultured islet beta cells. *Diabetologia* 2004;47:225-39.
201. Frisk G, Friman G, Tuvemo T, Fohlman J, Diderholm H. Coxsackie B virus IgM in children at onset of type 1 (insulin-dependent) diabetes mellitus: evidence for IgM induction by a recent or current infection. *Diabetologia* 1992;35:249-53.

202. Banatvala JE, Bryant J, Schernthaner G, et al. Coxsackie B, mumps, rubella, and cytomegalovirus specific IgM responses in patients with juvenile-onset insulin-dependent diabetes mellitus in Britain, Austria, and Australia. *Lancet* 1985;1:1409-12.
203. King ML, Shaikh A, Bidwell D, Voller A, Banatvala JE. Coxsackie-B-virus-specific IgM responses in children with insulin-dependent (juvenile-onset; type I) diabetes mellitus. *Lancet* 1983;1:1397-9.
204. Banatvala JE. Insulin-dependent (juvenile-onset, type 1) diabetes mellitus Coxsackie B viruses revisited. *Prog Med Virol* 1987;34:33-54.
205. Helfand RF, Gary HE, Jr., Freeman CY, Anderson LJ, Pallansch MA. Serologic evidence of an association between enteroviruses and the onset of type 1 diabetes mellitus. Pittsburgh Diabetes Research Group. *J Infect Dis* 1995;172:1206-11.
206. Rotbart H. PCR Protocols: A Guide to Methods and Application. PCR amplification of enterovirus. San Diego: Academic Press 1990:372-7.
207. Rotbart HA. Nucleic acid detection systems for enteroviruses. *Clin Microbiol Rev* 1991;4:156-68.
208. Clements GB, Galbraith DN, Taylor KW. Coxsackie B virus infection and onset of childhood diabetes. *Lancet* 1995;346:221-3.
209. Andreoletti L, Hober D, Hober-Vandenberghe C, et al. Detection of coxsackie B virus RNA sequences in whole blood samples from adult patients at the onset of type I diabetes mellitus. *J Med Virol* 1997;52:121-7.
210. Nairn C, Galbraith DN, Taylor KW, Clements GB. Enterovirus variants in the serum of children at the onset of Type 1 diabetes mellitus. *Diabet Med* 1999;16:509-13.
211. Oikarinen M, Tauriainen S, Honkanen T, et al. Detection of enteroviruses in the intestine of type 1 diabetic patients. *Clin Exp Immunol* 2008;151:71-5.
212. Vaarala O. Gut and the induction of immune tolerance in type 1 diabetes. *Diabetes Metab Res Rev* 1999;15:353-61.
213. Westerholm-Ormio M, Vaarala O, Pihkala P, Ilonen J, Savilahti E. Immunologic activity in the small intestinal mucosa of pediatric patients with type 1 diabetes. *Diabetes* 2003;52:2287-95.
214. Champsaur H, Dussaix E, Samolyk D, Fabre M, Bach C, Assan R. Diabetes and Coxsackie virus B5 infection. *Lancet* 1980;1:251.
215. Frisk G, Diderholm H. Antibody responses to different strains of coxsackie B4 virus in patients with newly diagnosed type I diabetes mellitus or aseptic meningitis. *J Infect* 1997;34:205-10.
216. Chehadeh W, Weill J, Vantghem MC, et al. Increased level of interferon-alpha in blood of patients with insulin-dependent diabetes mellitus: relationship with coxsackievirus B infection. *J Infect Dis* 2000;181:1929-39.
217. Cabrera-Rode E, Sarmiento L, Molina G, et al. Islet cell related antibodies and type 1 diabetes associated with echovirus 30 epidemic: a case report. *J Med Virol* 2005;76:373-7.

218. Hindersson M, Elshebani A, Orn A, Tuvemo T, Frisk G. Simultaneous type 1 diabetes onset in mother and son coincident with an enteroviral infection. *J Clin Virol* 2005;33:158-67.
219. Stene LC, Oikarinen S, Hyoty H, et al. Enterovirus Infection and Progression From Islet Autoimmunity to Type 1 Diabetes: The Diabetes and Autoimmunity Study in the Young (DAISY). *Diabetes*;59:3174-80.
220. Anderson MS, Bluestone JA. The NOD mouse: a model of immune dysregulation. *Annu Rev Immunol* 2005;23:447-85.
221. Crisa L, Mordes JP, Rossini AA. Autoimmune diabetes mellitus in the BB rat. *Diabetes Metab Rev* 1992;8:4-37.
222. Serreze DV, Ottendorfer EW, Ellis TM, Gauntt CJ, Atkinson MA. Acceleration of type 1 diabetes by a coxsackievirus infection requires a preexisting critical mass of autoreactive T-cells in pancreatic islets. *Diabetes* 2000;49:708-11.
223. Tracy S, Drescher KM. Coxsackievirus infections and NOD mice: relevant models of protection from, and induction of, type 1 diabetes. *Ann N Y Acad Sci* 2007;1103:143-51.
224. Houssay BA, Martinez C. Experimental Diabetes and Diet. *Science* 1947;105:548-9.
225. Stauffacher W, Burr I, Gutzeit A, Beaven D, Veleminsky J, Renold AE. Streptozotocin diabetes: time course of irreversible B-cell damage; further observations on prevention by nicotinamide. *Proc Soc Exp Biol Med* 1970;133:194-200.
226. Baum JD, Ounsted M, Smith MA. Letter: Weight gain in infancy and subsequent development of diabetes mellitus in childhood. *Lancet* 1975;2:866.
227. Blom L, Persson LA, Dahlquist G. A high linear growth is associated with an increased risk of childhood diabetes mellitus. *Diabetologia* 1992;35:528-33.
228. Dahlquist GG, Patterson C, Soltesz G. Perinatal risk factors for childhood type 1 diabetes in Europe. The EURODIAB Substudy 2 Study Group. *Diabetes Care* 1999;22:1698-702.
229. Rapid early growth is associated with increased risk of childhood type 1 diabetes in various European populations. *Diabetes Care* 2002;25:1755-60.
230. Hagopian WA, Lernmark A, Rewers MJ, et al. TEDDY--The Environmental Determinants of Diabetes in the Young: an observational clinical trial. *Ann N Y Acad Sci* 2006;1079:320-6.
231. Dahlquist G, Kallen B. Maternal-child blood group incompatibility and other perinatal events increase the risk for early-onset type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 1992;35:671-5.
232. Bingley PJ, Douek IF, Rogers CA, Gale EA. Influence of maternal age at delivery and birth order on risk of type 1 diabetes in childhood: prospective population based family study. Bart's-Oxford Family Study Group. *BMJ* 2000;321:420-4.
233. Wagener DK, LaPorte RE, Orchard TJ, Cavender D, Kuller LH, Drash AL. The Pittsburgh diabetes mellitus study. 3: An increased prevalence with older maternal age. *Diabetologia* 1983;25:82-5.

234. Patterson CC, Carson DJ, Hadden DR, Waugh NR, Cole SK. A case-control investigation of perinatal risk factors for childhood IDDM in Northern Ireland and Scotland. *Diabetes Care* 1994;17:376-81.
235. Mayer EJ, Hamman RF, Gay EC, Lezotte DC, Savitz DA, Klingensmith GJ. Reduced risk of IDDM among breast-fed children. The Colorado IDDM Registry. *Diabetes* 1988;37:1625-32.
236. Lindberg B, Ivarsson SA, Lernmark A. Islet autoantibodies in cord blood could be a risk factor for future diabetes. *Diabetologia* 1999;42:1375.
237. Eising S, Nilsson A, Carstensen B, et al. Danish children born 1981-2002 with GAD65 and IA-2 autoantibodies at birth had an increased risk to develop type 1 diabetes before 2004. *Eur J Endocrinol* 2010.
238. Ludvigsson J, Wahlberg J. Diabetes-related autoantibodies in cord blood from children of healthy mothers have disappeared by the time the child is one year old. *Ann N Y Acad Sci* 2002;958:289-92.
239. Rasmussen T, Stene LC, Samuelsen SO, et al. Maternal BMI before pregnancy, maternal weight gain during pregnancy, and risk of persistent positivity for multiple diabetes-associated autoantibodies in children with the high-risk HLA genotype: the MIDIA study. *Diabetes Care* 2009;32:1904-6.
240. Cardwell CR, Carson DJ, Patterson CC. Parental age at delivery, birth order, birth weight and gestational age are associated with the risk of childhood Type 1 diabetes: a UK regional retrospective cohort study. *Diabet Med* 2005;22:200-6.
241. Locatelli M, Buzzetti R, Galgani A, et al. Length of gestation and gender are associated with HLA genotypes at risk for Type 1 diabetes (Italian DIABFIN 3). *Diabet Med* 2007;24:916-9.
242. Stene LC, Magnus P, Lie RT, Sovik O, Joner G. Birth weight and childhood onset type 1 diabetes: population based cohort study. *BMJ* 2001;322:889-92.
243. Svensson J, Carstensen B, Mortensen HB, Borch-Johnsen K. Early childhood risk factors associated with type 1 diabetes--is gender important? *Eur J Epidemiol* 2005;20:429-34.
244. Stene LC, Magnus P, Lie RT, Sovik O, Joner G. No association between preeclampsia or cesarean section and incidence of type 1 diabetes among children: a large, population-based cohort study. *Pediatr Res* 2003;54:487-90.
245. Larsson HE, Hansson G, Carlsson A, et al. Children developing type 1 diabetes before 6 years of age have increased linear growth independent of HLA genotypes. *Diabetologia* 2008;51:1623-30.
246. Khan N, Couper JJ. Low-birth-weight infants show earlier onset of IDDM. *Diabetes Care* 1994;17:653-6.
247. Wahlberg J, Vaarala O, Ludvigsson J. Dietary risk factors for the emergence of type 1 diabetes-related autoantibodies in 2 1/2 year-old Swedish children. *Br J Nutr* 2006;95:603-8.
248. Virtanen SM, Rasanen L, Ylonen K, et al. Early introduction of dairy products associated with increased risk of IDDM in Finnish children. The Childhood in Diabetes in Finland Study Group. *Diabetes* 1993;42:1786-90.

249. Ziegler AG, Schmid S, Huber D, Hummel M, Bonifacio E. Early infant feeding and risk of developing type 1 diabetes-associated autoantibodies. *JAMA* 2003;290:1721-8.
250. Norris JM, Barriga K, Klingensmith G, et al. Timing of initial cereal exposure in infancy and risk of islet autoimmunity. *JAMA* 2003;290:1713-20.
251. Norris JM, Beaty B, Klingensmith G, et al. Lack of association between early exposure to cow's milk protein and beta-cell autoimmunity. Diabetes Autoimmunity Study in the Young (DAISY). *JAMA* 1996;276:609-14.
252. Helgason T, Jonasson MR. Evidence for a food additive as a cause of ketosis-prone diabetes. *Lancet* 1981;2:716-20.
253. Dahlquist GG, Blom LG, Persson LA, Sandstrom AI, Wall SG. Dietary factors and the risk of developing insulin dependent diabetes in childhood. *BMJ* 1990;300:1302-6.
254. Siemiatycki J, Colle E, Campbell S, Dewar RA, Belmonte MM. Case-control study of IDDM. *Diabetes Care* 1989;12:209-16.
255. Stene LC, Ulriksen J, Magnus P, Joner G. Use of cod liver oil during pregnancy associated with lower risk of Type I diabetes in the offspring. *Diabetologia* 2000;43:1093-8.
256. Vitamin D supplement in early childhood and risk for Type I (insulin-dependent) diabetes mellitus. The EURODIAB Substudy 2 Study Group. *Diabetologia* 1999;42:51-4.
257. Visalli N, Sebastiani L, Adorisio E, et al. Environmental risk factors for type 1 diabetes in Rome and province. *Arch Dis Child* 2003;88:695-8.
258. Essex MJ, Klein MH, Cho E, Kalin NH. Maternal stress beginning in infancy may sensitize children to later stress exposure: effects on cortisol and behavior. *Biol Psychiatry* 2002;52:776-84.
259. Sepa A, Frodi A, Ludvigsson J. Mothers' experiences of serious life events increase the risk of diabetes-related autoimmunity in their children. *Diabetes Care* 2005;28:2394-9.
260. Thernlund GM, Dahlquist G, Hansson K, et al. Psychological stress and the onset of IDDM in children. *Diabetes Care* 1995;18:1323-9.
261. Sepa A, Frodi A, Ludvigsson J. Could parenting stress and lack of support/confidence function as mediating mechanisms between certain environmental factors and the development of autoimmunity in children?: a study within ABIS. *Ann N Y Acad Sci* 2002;958:431-5.
262. Fujinami RS, Oldstone MB, Wroblewska Z, Frankel ME, Koprowski H. Molecular mimicry in virus infection: crossreaction of measles virus phosphoprotein or of herpes simplex virus protein with human intermediate filaments. *Proc Natl Acad Sci U S A* 1983;80:2346-50.
263. Hou J, Sheikh S, Martin DL, Chatterjee NK. Coxsackievirus B4 alters pancreatic glutamate decarboxylase expression in mice soon after infection. *J Autoimmun* 1993;6:529-42.
264. Atkinson MA, Bowman MA, Campbell L, Darrow BL, Kaufman DL, Maclaren NK. Cellular immunity to a determinant common to glutamate

- decarboxylase and coxsackie virus in insulin-dependent diabetes. *J Clin Invest* 1994;94:2125-9.
265. Kaufman DL, Erlander MG, Clare-Salzler M, Atkinson MA, Maclaren NK, Tobin AJ. Autoimmunity to two forms of glutamate decarboxylase in insulin-dependent diabetes mellitus. *J Clin Invest* 1992;89:283-92.
266. Hou J, Said C, Franchi D, Dockstader P, Chatterjee NK. Antibodies to glutamic acid decarboxylase and P2-C peptides in sera from coxsackie virus B4-infected mice and IDDM patients. *Diabetes* 1994;43:1260-6.
267. Richter W, Mertens T, Schoel B, et al. Sequence homology of the diabetes-associated autoantigen glutamate decarboxylase with coxsackie B4-2C protein and heat shock protein 60 mediates no molecular mimicry of autoantibodies. *J Exp Med* 1994;180:721-6.
268. Lehmann PV, Targoni OS, Forsthuber TG. Shifting T-cell activation thresholds in autoimmunity and determinant spreading. *Immunol Rev* 1998;164:53-61.
269. Soldevila G, Buscema M, Doshi M, James RF, Bottazzo GF, Pujol-Borrell R. Cytotoxic effect of IFN-gamma plus TNF-alpha on human islet cells. *J Autoimmun* 1991;4:291-306.
270. Cetkovic-Cvrlje M, Eizirik DL. TNF-alpha and IFN-gamma potentiate the deleterious effects of IL-1 beta on mouse pancreatic islets mainly via generation of nitric oxide. *Cytokine* 1994;6:399-406.
271. Tough DF, Sprent J. Viruses and T cell turnover: evidence for bystander proliferation. *Immunol Rev* 1996;150:129-42.
272. Jaeckel E, Manns M, Von Herrath M. Viruses and diabetes. *Ann N Y Acad Sci* 2002;958:7-25.
273. Horwitz MS, Bradley LM, Harbertson J, Krahl T, Lee J, Sarvetnick N. Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. *Nat Med* 1998;4:781-5.
274. Haring JS, Pewe LL, Perlman S. Bystander CD8 T cell-mediated demyelination after viral infection of the central nervous system. *J Immunol* 2002;169:1550-5.
275. von Herrath MG, Fujinami RS, Whitton JL. Microorganisms and autoimmunity: making the barren field fertile? *Nat Rev Microbiol* 2003;1:151-7.
276. Butz EA, Bevan MJ. Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. *Immunity* 1998;8:167-75.
277. Hislop AD, Annels NE, Gudgeon NH, Leese AM, Rickinson AB. Epitope-specific evolution of human CD8(+) T cell responses from primary to persistent phases of Epstein-Barr virus infection. *J Exp Med* 2002;195:893-905.
278. Welsh RM, Selin LK. No one is naive: the significance of heterologous T-cell immunity. *Nat Rev Immunol* 2002;2:417-26.
279. Murali-Krishna K, Altman JD, Suresh M, Sourdive D, Zajac A, Ahmed R. In vivo dynamics of anti-viral CD8 T cell responses to different epitopes. An evaluation of bystander activation in primary and secondary responses to viral infection. *Adv Exp Med Biol* 1998;452:123-42.

280. Strachan DP. Hay fever, hygiene, and household size. *BMJ* 1989;299:1259-60.
281. Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med* 2002;347:911-20.
282. Von Ehrenstein OS, Von Mutius E, Illi S, Baumann L, Bohm O, von Kries R. Reduced risk of hay fever and asthma among children of farmers. *Clin Exp Allergy* 2000;30:187-93.
283. Yemaneberhan H, Bekele Z, Venn A, Lewis S, Parry E, Britton J. Prevalence of wheeze and asthma and relation to atopy in urban and rural Ethiopia. *Lancet* 1997;350:85-90.
284. Feltbower RG, Bodansky HJ, McKinney PA, Houghton J, Stephenson CR, Haigh D. Trends in the incidence of childhood diabetes in south Asians and other children in Bradford, UK. *Diabet Med* 2002;19:162-6.
285. Serrano-Rios M, Goday A, Martinez Larrad T. Migrant populations and the incidence of type 1 diabetes mellitus: an overview of the literature with a focus on the Spanish-heritage countries in Latin America. *Diabetes Metab Res Rev* 1999;15:113-32.
286. Patterson CC, Carson DJ, Hadden DR. Epidemiology of childhood IDDM in Northern Ireland 1989-1994: low incidence in areas with highest population density and most household crowding. Northern Ireland Diabetes Study Group. *Diabetologia* 1996;39:1063-9.
287. Akira S, Kishimoto T. Mechanisms of soluble mediators. *Curr Opin Immunol* 1992;4:307-13.
288. Peters M. Actions of cytokines on the immune response and viral interactions: an overview. *Hepatology* 1996;23:909-16.
289. Zav'ialov VP. [The structural and functional classification and evolution of cytokines]. *Vestn Ross Akad Med Nauk* 1993;8-10.
290. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 1986;136:2348-57.
291. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 1996;17:138-46.
292. Dinarello CA. Historical insights into cytokines. *Eur J Immunol* 2007;37 Suppl 1:S34-45.
293. Guzeloglu-Kayisli O, Kayisli UA, Taylor HS. The role of growth factors and cytokines during implantation: endocrine and paracrine interactions. *Semin Reprod Med* 2009;27:62-79.
294. Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunol Today* 1993;14:353-6.
295. Athanassakis I, Vassiliadis S. Interplay between T helper type 1 and type 2 cytokines and soluble major histocompatibility complex molecules: a paradigm in pregnancy. *Immunology* 2002;107:281-7.
296. Chaouat G, Ledee-Bataille N, Dubanchet S, Zourbas S, Sandra O, Martal J. TH1/TH2 paradigm in pregnancy: paradigm lost? Cytokines in

- pregnancy/early abortion: reexamining the TH1/TH2 paradigm. *Int Arch Allergy Immunol* 2004;134:93-119.
297. Ashkar AA, Di Santo JP, Croy BA. Interferon gamma contributes to initiation of uterine vascular modification, decidual integrity, and uterine natural killer cell maturation during normal murine pregnancy. *J Exp Med* 2000;192:259-70.
298. Fukushima K, Miyamoto S, Tsukimori K, et al. Tumor necrosis factor and vascular endothelial growth factor induce endothelial integrin repertoires, regulating endovascular differentiation and apoptosis in a human extravillous trophoblast cell line. *Biol Reprod* 2005;73:172-9.
299. Veith GL, Rice GE. Interferon gamma expression during human pregnancy and in association with labour. *Gynecol Obstet Invest* 1999;48:163-7.
300. Hebisch G, Neumaier-Wagner PM, Huch R, von Mandach U. Maternal serum interleukin-1 beta, -6 and -8 levels and potential determinants in pregnancy and peripartum. *J Perinat Med* 2004;32:475-80.
301. Opsjln SL, Wathen NC, Tingulstad S, et al. Tumor necrosis factor, interleukin-1, and interleukin-6 in normal human pregnancy. *Am J Obstet Gynecol* 1993;169:397-404.
302. Hill JA, Polgar K, Anderson DJ. T-helper 1-type immunity to trophoblast in women with recurrent spontaneous abortion. *JAMA* 1995;273:1933-6.
303. Daher S, de Arruda Geraldes Denardi K, Blotta MH, et al. Cytokines in recurrent pregnancy loss. *J Reprod Immunol* 2004;62:151-7.
304. Saito S, Umekage H, Sakamoto Y, et al. Increased T-helper-1-type immunity and decreased T-helper-2-type immunity in patients with preeclampsia. *Am J Reprod Immunol* 1999;41:297-306.
305. Saito S. Cytokine network at the feto-maternal interface. *J Reprod Immunol* 2000;47:87-103.
306. Lin H, Mosmann TR, Guilbert L, Tuntipopipat S, Wegmann TG. Synthesis of T helper 2-type cytokines at the maternal-fetal interface. *J Immunol* 1993;151:4562-73.
307. Wilczynski JR. Th1/Th2 cytokines balance--yin and yang of reproductive immunology. *Eur J Obstet Gynecol Reprod Biol* 2005;122:136-43.
308. Mikloska Z, Cunningham AL. Alpha and gamma interferons inhibit herpes simplex virus type 1 infection and spread in epidermal cells after axonal transmission. *J Virol* 2001;75:11821-6.
309. Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. *Annu Rev Immunol* 1997;15:749-95.
310. Guidotti LG, Chisari FV. Noncytolytic control of viral infections by the innate and adaptive immune response. *Annu Rev Immunol* 2001;19:65-91.
311. Chen SH, Oakes JE, Lausch RN. Synergistic anti-HSV effect of tumor necrosis factor alpha and interferon gamma in human corneal fibroblasts is associated with interferon beta induction. *Antiviral Res* 1993;22:15-29.
312. Trinchieri G. Interleukin-12 and its role in the generation of TH1 cells. *Immunol Today* 1993;14:335-8.



313. Malmgaard L, Paludan SR. Interferon (IFN)-alpha/beta, interleukin (IL)-12 and IL-18 coordinately induce production of IFN-gamma during infection with herpes simplex virus type 2. *J Gen Virol* 2003;84:2497-500.
314. Ulich TR, del Castillo J, Keys M, Granger GA, Ni RX. Kinetics and mechanisms of recombinant human interleukin 1 and tumor necrosis factor-alpha-induced changes in circulating numbers of neutrophils and lymphocytes. *J Immunol* 1987;139:3406-15.
315. Gery I, Gershon RK, Waksman BH. Potentiation of the T-lymphocyte response to mitogens. I. The responding cell. *J Exp Med* 1972;136:128-42.
316. Smith KA. Interleukin-2: inception, impact, and implications. *Science* 1988;240:1169-76.
317. Street NE, Mosmann TR. Functional diversity of T lymphocytes due to secretion of different cytokine patterns. *FASEB J* 1991;5:171-7.
318. Gogos CA, Drosou E, Bassaris HP, Skoutelis A. Pro- versus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options. *J Infect Dis* 2000;181:176-80.
319. Walker-Smith J GS, Schmitz J, Shmerling D, Visakorpi J. Revised criteria for diagnosis of coeliac disease. Report of Working Group of European Society of Paediatric Gastroenterology and Nutrition. *Arch Dis Child* 1990;65:909-11.
320. Stenhammar L, Ansved P, Jansson G, Jansson U. The incidence of childhood coeliac disease in Sweden. *J Pediatr Gastroenterol Nutr* 1987;6:707-9.
321. Cavell B, Stenhammar L, Ascher H, et al. Increasing incidence of childhood coeliac disease in Sweden. Results of a national study. *Acta Paediatr* 1992;81:589-92.
322. Ivarsson A, Persson LA, Nystrom L, et al. Epidemic of coeliac disease in Swedish children. *Acta Paediatr* 2000;89:165-71.
323. Farrell RJ, Kelly CP. Celiac sprue. *N Engl J Med* 2002;346:180-8.
324. Sollid LM, Markussen G, Ek J, Gjerde H, Vartdal F, Thorsby E. Evidence for a primary association of coeliac disease to a particular HLA-DQ alpha/beta heterodimer. *J Exp Med* 1989;169:345-50.
325. Sollid LM. Molecular basis of coeliac disease. *Annu Rev Immunol* 2000;18:53-81.
326. Mazzarella G, Maglio M, Paparo F, et al. An immunodominant DQ8 restricted gliadin peptide activates small intestinal immune response in in vitro cultured mucosa from HLA-DQ8 positive but not HLA-DQ8 negative coeliac patients. *Gut* 2003;52:57-62.
327. Greco L, Romino R, Coto I, et al. The first large population based twin study of coeliac disease. *Gut* 2002;50:624-8.
328. Dieterich W, Ehnis T, Bauer M, et al. Identification of tissue transglutaminase as the autoantigen of coeliac disease. *Nat Med* 1997;3:797-801.
329. Butt S. New clues point to coeliac disease. *Practitioner* 1999;243:276, 9-82.
330. Sandberg-Bennich S, Dahlquist G, Kallen B. Coeliac disease is associated with intrauterine growth and neonatal infections. *Acta Paediatr* 2002;91:30-3.

331. Ivarsson A, Hernell O, Nystrom L, Persson LA. Children born in the summer have increased risk for coeliac disease. *J Epidemiol Community Health* 2003;57:36-9.
332. Maki M, Hallstrom O, Huupponen T, Vesikari T, Visakorpi JK. Increased prevalence of coeliac disease in diabetes. *Arch Dis Child* 1984;59:739-42.
333. Cronin CC, Shanahan F. Insulin-dependent diabetes mellitus and coeliac disease. *Lancet* 1997;349:1096-7.
334. Bao F, Yu L, Babu S, et al. One third of HLA DQ2 homozygous patients with type 1 diabetes express celiac disease-associated transglutaminase autoantibodies. *J Autoimmun* 1999;13:143-8.
335. Bonifacio E, Ziegler AG, Hummel M, et al. Gluten: is it also a determinant of islet autoimmunity? *Diabetes Metab Rev* 1998;14:258-9.
336. Cerutti F, Bruno G, Chiarelli F, Lorini R, Meschi F, Sacchetti C. Younger age at onset and sex predict celiac disease in children and adolescents with type 1 diabetes: an Italian multicenter study. *Diabetes Care* 2004;27:1294-8.
337. Larsson K, Elding-Larsson H, Cederwall E, et al. Genetic and perinatal factors as risk for childhood type 1 diabetes. *Diabetes Metab Res Rev* 2004;20:429-37.
338. Lernmark B, Elding-Larsson H, Hansson G, Lindberg B, Lynch K, Sjoblad S. Parent responses to participation in genetic screening for diabetes risk. *Pediatr Diabetes* 2004;5:174-81.
339. Larsson HE, Lynch K, Lernmark B, et al. Diabetes-associated HLA genotypes affect birthweight in the general population. *Diabetologia* 2005;48:1484-91.
340. Björck S, Brundin, C., Lörinc, E., Lynch, K. F., Agardh, D. Screening Detects A High Proportion of Celiac Disease in Young HLA-Genotyped Children. *Journal of Pediatric Gastroenterology and Nutrition* 2009;49:1-6.
341. Ryding J, French KM, Naucler P, Barnabas RV, Garnett GP, Dillner J. Seroepidemiology as basis for design of a human papillomavirus vaccination program. *Vaccine* 2008;26:5263-8.
342. Sjoroos M, Iitia A, Ilonen J, Reijonen H, Lovgren T. Triple-label hybridization assay for type-1 diabetes-related HLA alleles. *Biotechniques* 1995;18:870-7.
343. Ilonen J, Reijonen H, Herva E, et al. Rapid HLA-DQB1 genotyping for four alleles in the assessment of risk for IDDM in the Finnish population. The Childhood Diabetes in Finland (DiMe) Study Group. *Diabetes Care* 1996;19:795-800.
344. Hampe CS, Hammerle LP, Bekris L, et al. Recognition of glutamic acid decarboxylase (GAD) by autoantibodies from different GAD antibody-positive phenotypes. *J Clin Endocrinol Metab* 2000;85:4671-9.
345. Williams AJ, Bingley PJ, Bonifacio E, Palmer JP, Gale EA. A novel micro-assay for insulin autoantibodies. *J Autoimmun* 1997;10:473-8.
346. Torn C, Mueller PW, Schlosser M, Bonifacio E, Bingley PJ. Diabetes Antibody Standardization Program: evaluation of assays for autoantibodies to glutamic acid decarboxylase and islet antigen-2. *Diabetologia* 2008;51:846-52.

347. Germer JJ, Lins MM, Jensen ME, et al. Evaluation of the MagNA pure LC instrument for extraction of hepatitis C virus RNA for the COBAS AMPLICOR Hepatitis C Virus Test, version 2.0. *J Clin Microbiol* 2003;41:3503-8.
348. Romero JR. Reverse-transcription polymerase chain reaction detection of the enteroviruses. *Arch Pathol Lab Med* 1999;123:1161-9.
349. Oberste MS, Penaranda S, Rogers SL, Henderson E, Nix WA. Comparative evaluation of Taqman real-time PCR and semi-nested VP1 PCR for detection of enteroviruses in clinical specimens. *J Clin Virol*;49:73-4.
350. Kilpatrick DR, Yang CF, Ching K, et al. Rapid group-, serotype-, and vaccine strain-specific identification of poliovirus isolates by real-time reverse transcription-PCR using degenerate primers and probes containing deoxyinosine residues. *J Clin Microbiol* 2009;47:1939-41.
351. Lindehammer SR, Bj Rck S, Lynch K, et al. Early human pregnancy serum cytokine levels predict autoimmunity in offspring. *Autoimmunity* 2011.
352. D'Armiento M, Salabe H, Vetrano G, Scucchia M, Pachi A. Decrease of thyroid antibodies during pregnancy. *J Endocrinol Invest* 1980;3:437-8.
353. Wilder RL. Hormones, pregnancy, and autoimmune diseases. *Ann N Y Acad Sci* 1998;840:45-50.
354. Papadopoulou A, Lynch KF, Shaat N, et al. The type 1 diabetes protective HLA DQB1\*0602 allele is less frequent in gestational diabetes mellitus. *Diabetologia* 2009;52:1339-42.
355. Fronczak CM, Baron AE, Chase HP, et al. In utero dietary exposures and risk of islet autoimmunity in children. *Diabetes Care* 2003;26:3237-42.
356. Bonifacio E, Lernmark A, Dawkins RL. Serum exchange and use of dilutions have improved precision of measurement of islet cell antibodies. *J Immunol Methods* 1988;106:83-8.
357. Chowdhury F, Williams A, Johnson P. Validation and comparison of two multiplex technologies, Luminex and Mesoscale Discovery, for human cytokine profiling. *J Immunol Methods* 2009;340:55-64.
358. Wong HL, Pfeiffer RM, Fears TR, Vermeulen R, Ji S, Rabkin CS. Reproducibility and correlations of multiplex cytokine levels in asymptomatic persons. *Cancer Epidemiol Biomarkers Prev* 2008;17:3450-6.
359. Flower L, Ahuja RH, Humphries SE, Mohamed-Ali V. Effects of sample handling on the stability of interleukin 6, tumour necrosis factor-alpha and leptin. *Cytokine* 2000;12:1712-6.
360. Thavasu PW, Longhurst S, Joel SP, Slevin ML, Balkwill FR. Measuring cytokine levels in blood. Importance of anticoagulants, processing, and storage conditions. *J Immunol Methods* 1992;153:115-24.
361. Aziz N, Nishanian P, Mitsuyasu R, Detels R, Fahey JL. Variables that affect assays for plasma cytokines and soluble activation markers. *Clin Diagn Lab Immunol* 1999;6:89-95.
362. Mohamed-Ali V, Goodrick S, Rawesh A, et al. Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-alpha, in vivo. *J Clin Endocrinol Metab* 1997;82:4196-200.

363. Barrett-Connor E. Is insulin-dependent diabetes mellitus caused by coxsackievirus B infection? A review of the epidemiologic evidence. *Rev Infect Dis* 1985;7:207-15.