Olli Helminen

GLUCOSE METABOLISM IN PRECLINICAL TYPE 1 DIABETES
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Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium 12 of Oulu University Hospital (Kajaanintie 50), on 7 October 2016, at 1 p.m

UNIVERSITY OF OULU, OULU 2016
Abstract

Type 1 diabetes is considered to be a T cell-mediated autoimmune disease characterized by destruction of the pancreatic beta cells. Its prediction is currently based on diabetes-associated autoantibodies, giving a cumulative risk of 84% during 15 years of follow-up since seroconversion. Prediction of the timing of clinical onset has remained challenging, however. This thesis examines glucose metabolism in autoantibody-positive children with a high risk of developing type 1 diabetes. Out of a total of 14,876 children with an increased genetic risk followed up from birth in the Finnish DIPP study, 567 developed ≥2 autoantibodies during the follow-up and 255 of these (45%) were diagnosed with type 1 diabetes until the end of December 2011. The glucose parameters measured were HbA1c, OGTT and random plasma glucose with 3 to 12 months interval. Seven-day continuous glucose monitoring (CGM) was performed on an age and sex-matched cohort. We showed that rising HbA1c, impaired glucose tolerance in OGTT, random plasma glucose values of ≥7.8mmol/l and potentially CGM can predict type 1 diabetes with a median time to diagnosis of approximately one year. Our results suggest that especially HbA1c and random plasma glucose are cost-effective and improve the prediction of diabetes. These markers may be useful for monitoring the response to treatment in prevention studies.

Keywords: autoimmunity, C-peptide, continuous glucose monitoring, dysglycemia, glucose intolerance, HbA1c, HLA, islet autoantibodies, oral glucose tolerance test, prediabetes, self-monitoring blood glucose, type 1 diabetes
Helminen, Olli, Sokeriaineenvaihdunta preklinisessä tyypin 1 diabeteksessa.
Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta; Medical Research Center Oulu; Oulun yliopistollinen sairaala
Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

Tiivistelmä


Asiasanat: C-peptidi, jatkuva sokeripitoisuuden seuranta, sokeriaineenvaihdunnan poikkeavuus, glukoosi-intoleranssi, HbA1c, HLA, autovasta-aineet, oraalinen sokerirasituskoe, prediabetes, verensokerin kotimittaus, tyypin 1 diabetes
Acknowledgements

This study was carried out at the Department of Pediatrics, University of Oulu, and Oulu University Hospital, Oulu, Finland.

I wish to thank my supervisor, Professor Riitta Veijola, for leading me into the world of science with all its good and challenging aspects. I am grateful to her for giving me this opportunity and for making the work possible.

It goes without saying that high-quality research is possible only with productive teamwork and collaboration. My special thanks go to Tytti Pokka, M.Sc., for her immeasurably valuable statistical expertise and for sharing the attendant labour pains and setbacks with a constantly positive attitude. I am grateful to all the co-authors and collaborators at the Universities of Oulu, Tampere, Turku and Helsinki, especially Professor Mikael Knip for his enthusiastic approach to research. Similarly, I wish to thank Professor Jorma Ilonen for contributing expertise in genetics and Professor Olli Simell for trusting me with the data. I also owe a debt of gratitude to Susanna Aspholm, Nora Haatanen, Johanna Lempainen and Päivi Tossavainen, and I similarly wish to express my gratitude to the staff of the DIPP study, the Outpatient Pediatric Diabetes Clinic and ward 62/2 for their collaboration.

I wish to acknowledge the importance of my research group at the Departments of Pathology, Surgery and Anatomy & Cell Biology, led by Professor Tuomo Karttunen, Professor Petri Lehenkari, Docent Juha Saarnio and Docent Joonas Kauppila. In particular I would thank Tuomo for his guidance and for being a role model in matters of efficiency, Petri for his contagious enthusiasm for research and Juha for his support that always inspires people to give their best. My special thanks go to Heikki Hultta, who really showed me that, after all, this job is a lot of fun!

I warmly thank the department heads, Chief Physician Päivi Tapanainen, Professor Matti Uhari, Professor Mikko Hallman and Professor Mika Rämet, for creating an encouraging and inspiring atmosphere for scientific work.

I wish to acknowledge Professors Jussi Pihlajamäki and Harri Niinikoski for their careful revision of this thesis and for their valuable comments. I thank Malcolm Hicks for his careful language review of the thesis, and I am grateful to Docent Leena Moilanen for accepting the role of official opponent in the eventual discussion of the thesis.

At the same time I would like to thank my many dear friends for taking my mind off work and research from time to time in the form of vacations, sports and get-togethers. I would especially like to mention Juuso Heikkinen, Samuli
Juntunen, Sanna Huhtaniska, Pekka Peroja, Sami Palomäki, Liisa Harjama and Heikki Rautio. I am grateful to my family for all their support and for showing me that learning is a lifelong process to be enjoyed wholeheartedly. And last but not least, I am happy that I can share and enjoy research and life in general with my dear Milla for years to come.

This study was supported financially by the Jalmari and Rauha Ahokas Foundation, the Finnish Cultural Foundation’s North Ostrobothnia Regional Fund, the Diabetes Research Foundation, the Finnish Medical Foundation, the Emil Aaltonen Foundation and the Alma and K.A. Snellman Foundation.
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<td>ADA</td>
<td>American Diabetes Association</td>
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<tr>
<td>BBDP</td>
<td>biobreeding diabetes–prone</td>
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<td>BMI</td>
<td>body mass index</td>
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<td>CGM</td>
<td>continuous glucose monitoring</td>
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<td>CTLA4</td>
<td>cytotoxic T–lymphocyte–associated protein 4</td>
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<tr>
<td>DAISY</td>
<td>Diabetes and Autoimmunity Study in the Young</td>
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<td>DIPP</td>
<td>Type 1 Diabetes Prediction and Prevention</td>
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<td>DPT–1</td>
<td>Diabetes Prevention Trial–Type 1</td>
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<tr>
<td>FOXp3</td>
<td>forkhead box transcription factor</td>
</tr>
<tr>
<td>FDR</td>
<td>first–degree relative</td>
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<td>FPIR</td>
<td>first phase insulin response</td>
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<td>FPLC</td>
<td>fast protein liquid chromatography</td>
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<td>GADA</td>
<td>glutamic acid decarboxylase antibodies</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
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<tr>
<td>HOMA–IR</td>
<td>homeostasis modes assessment of insulin resistance</td>
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<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>HR</td>
<td>hazard ratio</td>
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<td>IA–2A</td>
<td>islet antigen 2 antibodies</td>
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<tr>
<td>IAA</td>
<td>insulin autoantibodies</td>
</tr>
<tr>
<td>ICA</td>
<td>islet cell antibodies</td>
</tr>
<tr>
<td>IFG</td>
<td>impaired fasting glucose</td>
</tr>
<tr>
<td>IFIH1</td>
<td>interferon induced with helicase C domain</td>
</tr>
<tr>
<td>IgM</td>
<td>immunoglobulin M</td>
</tr>
<tr>
<td>IGT</td>
<td>impaired glucose tolerance</td>
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<tr>
<td>IL2RA</td>
<td>interleukin 2 receptor alpha</td>
</tr>
<tr>
<td>IPEX</td>
<td>immune dysfunction, polyendocrinopathy, enteropathy, X–linked inheritance</td>
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<td>IVGTT</td>
<td>intravenous glucose tolerance test</td>
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<tr>
<td>JDRF</td>
<td>Juvenile Diabetes Research Foundation</td>
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<tr>
<td>MAGE</td>
<td>mean amplitude of glycaemic excursion</td>
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<td>Md</td>
<td>median</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
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<td>NOD</td>
<td>non–obese diabetic</td>
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<tr>
<td>nPOD</td>
<td>Network for Pancreatic Organ Donors with Diabetes</td>
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<tr>
<td>OGTT</td>
<td>oral glucose tolerance test</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PTPN22</td>
<td>protein tyrosine phosphatase non–receptor type 22</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SMBG</td>
<td>self–monitored blood glucose</td>
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<tr>
<td>SNP</td>
<td>single–nucleotide polymorphism</td>
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<tr>
<td>T1D</td>
<td>type 1 diabetes</td>
</tr>
<tr>
<td>T1DGC</td>
<td>Type 1 Diabetes Genetic Consortium</td>
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<tr>
<td>TAP</td>
<td>transporter associated with antigen processing</td>
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<td>TEDDY</td>
<td>The Environmental Determinants of Diabetes in the Young</td>
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<tr>
<td>Treg</td>
<td>regulatory T cell</td>
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<tr>
<td>TRIGR</td>
<td>Trial to Reduce IDDM in the Genetically at Risk</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>ZnT8A</td>
<td>zinc transporter family member 8 antibodies</td>
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List of original publications

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1 Introduction

Type 1 diabetes is one of the most common chronic diseases in childhood, and its incidence has been rising throughout the western world. In Finland it has more than doubled from 1980 to 2005 in children under the age of 15 years, although encouraging signs of a plateau in the incidence rates have been observed recently. Presentation with type 1 diabetes is typically acute, although the decline in beta cell function begins several years before the appearance of symptoms. The development of type 1 diabetes is characterized by T cell–mediated selective destruction of the pancreatic beta cells, which eventually eliminates insulin production. The pathogenesis results from interactions between both genetic and environmental factors. Genetic risk factors associated with human histocompatibility leukocyte antigen (HLA) and more than 50 other genes have been well documented, but no single environmental risk factor has yet been identified.

In children with genetic predisposition an as yet unidentified trigger initiates the immune response. The development of islet autoantibodies can be observed in peripheral blood samples and the prediction of type 1 diabetes is based on autoantibodies which provide relatively good accuracy. Currently five diabetes–associated autoantibodies are considered most significant: islet cell antibodies (ICA), insulin autoantibodies (IAA), glutamic acid decarboxylase antibodies (GADA), islet antigen 2 antibodies (IA–2A) and zinc transporter 8 antibodies (ZnT8A). Individuals with ≥2 autoantibodies have a cumulative risk of 40–50% over the next 5 years, and the risk of developing diabetes within 15 years is over 80%.

Even so, there is a lack of a proper way of predicting the timing of incident type 1 diabetes. This is particularly important in order to prevent the occurrence of acute and possibly life-threatening complications. The present study was designed to characterize glucose metabolism in preclinical type 1 diabetes, and to provide accurate methods for predicting the timing of the clinical disease.
2 Review of the literature

2.1 History of Type 1 diabetes

Medical condition characterized by thirst, continuous urination and weight loss with lethal outcome has been described by medical authors for over three millennia. First written case report, obtained by the Egyptologist Georg Ebers in 1874, dated back to 1500 BCE describing a condition of “too great emptying of the urine” (1). Around the same era Indian physicians developed the first clinical test for diagnosing diabetes. They observed that some people’s urine attracted ants and flies, and named the condition “honey urine”. Along with this sweet urine, patients suffering from this condition had continuous extreme thirst and foul breath, suggesting ketoacidosis (1). Later the term “diabetes” came into use in 250 BCE, originally meaning “to pass through”. Type 1 and type 2 diabetes were identified as separate conditions for the first time in India around 400–500 CE by two physicians who suggested that type 1 appeared in thin, young individuals, whereas heavier people acquired type 2 diabetes and lived longer after the diagnosis. The treatment, however, still varied from advocating or avoiding sweet food to bloodletting, dehydration and starvation, leading invariably to death (2).

The origin of today’s understanding of diabetes can be traced to observations made in Europe between the 16th and 18th centuries. The famous Swiss physician Paracelsus (1494–1541) evaporated and observed the white residue in urine samples obtained from patients with typical symptoms, but incorrectly believed it to be salt (2). Later, the British physiologist Matthew Dobson (1713–1784) not only identified this white substance as sugar in 1776, but also noticed a sweet taste in serum samples, thus discovering hyperglycaemia (2). People with traumatic injury to the pancreas often developed diabetes and an association between the two was suggested for the first time in 1788.

Along with modern scientific disciplines, improvements in experimental physiology shed some light on the mystery of diabetes. Along with the experimental studies of the gastrointestinal tract, Claude Bernard (1813–1878), a professor of physiology at the Sorbonne, ligated the pancreatic ducts, causing degeneration and damage to the pancreas and leading to hyperglycaemia. This still did not reveal the pancreatic substance that reduced glucose levels, however, since Dr. Bernard simultaneously found glycogen stored in the liver and thought that its
excess secretion caused diabetes. The theory gained wide acceptance among physicians (2).

Despite the advances in characterizing the disease, its prognosis remained the same from antiquity up to the 20th century, until small steps were made towards a breakthrough in the discovery of insulin (3).

Oscar Minkowski (1858–1931) and Joseph von Mering (1849–1908) carried out experiments with dogs in Strasbourg in 1889 in which they removed the pancreas, causing symptoms typical of diabetes. Attempts to extract the crucial pancreatic substance involved were unsuccessful, however (4). Pancreatectomy immediately caused the appropriate symptoms, but it was still unclear whether the key substance was secreted in the gut or endogenously. Minkowski then performed an animal experiment in which, after pancreatectomy, he grafted a piece of tissue that had been removed under the skin, and no evidence of diabetes was present in the animals at this stage. It became clear that the glucose–lowering agent had been secreted internally (4). In 1910 this substance was named insulin, from the Latin insula, after Langerhans’ islets. Numerous scientists made efforts to obtain purified insulin, resulting in intolerable complications in the treatment.

In 1921 a young orthopedic surgeon named Frederick Grant Banting (1891–1941) managed to harvest atrophied glands from a dog pancreas after ligation of the pancreatic duct, chopped them up, grounded the tissue, strained the solution and injected it to the vein of a dog on which a pancreatectomy had been performed. The dog’s condition improved dramatically. After multiple experiments and assistance from the biochemist James Collip (1892–1965), insulin was injected into a 14–year–old boy with type 1 diabetes. His urinary ketones disappeared and the boy lived for another 13 years (5). The commercial production of insulin started the same year and the discovery earned Banting and the group leader, John J.R. MacLeod (1876–1935), a Nobel Prize in 1923.

Nearly a century has passed since the isolation of insulin, and despite the development of synthetic human insulin, novel insulin pumps and continuous monitoring, the life expectancy of patients with type 1 diabetes is still more than 10 years shorter than that of age and sex–matched controls (6). Also, no breakthroughs have been achieved in the prevention of the disease (7). The finding of efficacious and safe means of preventing type 1 diabetes will be a great challenge for the future.
2.1.1 Pathophysiology of type 1 diabetes

The hyperglycaemia that occurs in type 1 diabetes is a consequence of a deficiency of insulin–producing beta cells in the pancreas (8). The selective destruction of beta cells is regarded as an archetypal example of a T cell–mediated autoimmune disease. In this classical model individuals encounter a factor, possibly a viral infection (9–12) or predisposing antigen (13) that triggers primary damage to the beta cells. Dendritic antigen–presenting cells are activated, acquiring autoantigens in the damaged area of the pancreas (14). These cells then migrate to local lymph nodes, presenting an autoantigen to the T cells. In individuals with risk class II human histocompatibility leukocyte antigen (HLA) alleles this triggers the activation and production of autoreactive effector CD4 and CD8 T cells. T cell migration to the Langerhans islets occurs, causing an inflammatory response that was already known in 1940 as “insulitis” (15), and the destruction of beta cells. This autoimmune process can be detected in peripheral blood with autoantibodies to beta cell structures (16). When 85–90% of the pancreatic beta cells have perished, the classic symptoms of polyuria, polydipsia and fatigue occur (8). Carbohydrate metabolism can be restored with insulin therapy, after which the patients are dependent on exogenous insulin for the rest of their lives.

2.2 Epidemiology of type 1 diabetes

Type 1 diabetes is one of the most common diseases in childhood and is being diagnosed at an increasing rate in adults as well. Its incidence varies geographically, with Finland having the highest figure in 2006, 64.3 per 100,000 person–years in children aged 0–14 years (17), followed by Sweden, Norway, Sardinia and the United Kingdom all with age–adjusted rates of over 20 per 100,000 person–years. The lowest incidences of type 1 diabetes have been reported in China and South America, with less than 1 case per 100,000 patient–years (18). The rate is increasing in most countries, especially in the western world, with the effect centring on children under the age of 5 years (19). Similar observations have been made in Finland, although the peak incidence in 2006 has been followed by a plateau (17). The annual increase from 1990 to 1999 was 2.8% worldwide (18), and more recent data for Europe extending up to 2008 show a 3.4% rise per annum (19), although as in Finland, the increase has not been uniform, showing periods of less and more rapid rises in the incidence. Further time trend analyses will show whether the peak has really been reached.
Unlike most autoimmune diseases, type 1 diabetes is more common in boys, with approximately a 1.2–fold risk (17). Approximately 1 individual per 100 in the general population of Finland is affected by type 1 diabetes. Familial clustering has been described in numerous studies, the risk of developing the disease being 8 to 15–fold higher in first–degree relatives (20, 21) and approximately two–fold even in second–degree relatives (20, 22). Since the overall prevalence of type 1 diabetes is 0.3% to 1% depending on the geographical location, the majority of children are diagnosed with the sporadic form of diabetes. The proportion of children with an affected first–degree relative at the time of diagnosis is 10–12% (23, 24), increasing with a long follow–up (25). Interestingly, the offspring of fathers with type 1 diabetes transmit the disease to 4–7% of their children, whereas the children of affected mothers have only a 1.5–3% probability of developing diabetes (26, 27). When considering extended family histories including first and second–degree relatives, approximately 20% of children with newly diagnosed type 1 diabetes have an affected person in the family (28).

2.3 Genetic susceptibility

Four decades of study have shown that immune genes, especially those that encode the major histocompatibility complex (MHC; HLA in humans), confer a risk of type 1 diabetes. The first evidence for an association between HLA and type 1 diabetes was found in 1973, when the frequency of the HLA class I alleles B8 and B15 was higher in patients with type 1 diabetes than in non–affected controls (29, 30). Family studies followed, confirming the association with the HLA region (31). Later studies have found multiple genetic determinants of susceptibility to type 1 diabetes, with the HLA region as the most significant (32, 33). Risk HLA alleles are present in approximately 50% of individuals with type 1 diabetes, whereas their prevalence in the general population is around 10% (33, 34). Logical hypotheses involving molecular interaction between the T cell receptor and the HLA–peptide complex at the beginning of autoimmunity have been made (35) but are yet to be confirmed.

2.3.1 The MHC (major histocompatibility complex) region

The MHC region is the most important region in the vertebrate genome with regard to infections and autoimmunity, and is also crucial for adaptive and innate immunity (36). The HLA complex is located on chromosome 6 and contains over
200 genes, of which more than 40 encode leukocyte antigens (37). The genes involved in immune response are divided into two categories, HLA I and II, which differ in structure and function. Genetic complexity and possible malfunction of the HLA system causes wide-ranging effects in immune responses and further clinical disorders.

**HLA structure, genes and function**

Class I genes encode the α polypeptide chain of the class I molecule. The genes coding for the β chain are located in chromosome 15. The α chain consists of 5 domains, two peptide–binding, one immunoglobulin–like, a transmembrane region, and the cytoplasmic tail. The three immunologically most important out of the 20 class I genes in the HLA region are HLA–A, B and C (Fig. 1). Class I genes are expressed by most somatic cells (38).

The α and β polypeptide chains of the HLA II molecule are encoded by class II genes (Fig. 1). The nomenclature of these genes consist of three letters, the first (D) indicating the class, the second (M, O, P, Q, R) the family, and the third (A, B) the α or β chain. The α and β chains each have four domains: the peptide–binding domain, the immunoglobulin–like domain, the transmembrane region and the cytoplasmic tail. Unlike class I, class II molecules are expressed mainly in immune cells, including B cells, activated T cells, macrophages, dendritic cells and thymic epithelial cells (39).

The HLA class III molecules include several secreted proteins of importance for immune functions, such as the complement system, cytokines and heat shock proteins (Fig. 1). These, along with the transporter associated with antigen processing (TAP), which is encoded by HLA class I genes, have a role in transporting ingested and fragmented proteins (self–derived or pathogen–derived) to the transmembrane HLA molecules to be loaded and presented in the peptide–binding groove (39–41).

Since the processing of thousands of self–derived old, worn–out proteins and the phagocytosis of pathogens is continuous, the consequence is that the surfaces of the cells are covered with approximately 100,000 to 300,000 class I or class II products. Vast numbers of antigens are presented, and these are eventually recognized by the T cells (42, 43), differentiating either to helper (CD4) or cytotoxic killer (CD8) T cells (44–46). Only the most strongly self–reactive T cells are deleted in the thymus, and marginally autoreactive cells are controlled by mostly unidentified mechanisms. When these mechanisms fail, self–reactive T cells
can be activated by certain HLA molecules, leading to autoimmune disorders (47, 48).

![Diagram of the HLA region in chromosome 6p, modified from Mehers and Gillespie (49).]

**HLA association with autoimmune diseases**

The studies of HLA association with autoimmune diseases that took place in the 1970s were prompted by the observation of association of HLA–B*27 with ankylosing spondylitis (50). HLA connections were then observed with several other autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, coeliac disease, systemic lupus erythematosus and also type 1 diabetes (30, 51–54).

**HLA association with type 1 diabetes**

The earliest report of an HLA association with type 1 diabetes came to light in 1973 and 1974 (29, 30) with the discovery of the HL–A antigen, known today as HLA–DRB1*04:01 and of HL–A8, corresponding to HLA–B*08:01. These associations were later confirmed in family–based studies (55).

**HLA class I and type 1 diabetes**

HLA I molecules present antigens to cytotoxic (CD8) T cells, which are thought to contribute to insulitis and beta cell killing in the pancreas, so that the association of HLA class I with susceptibility to type 1 diabetes is biologically logical. High–
powered studies have later proved this connection, even after accounting for other predisposing genes (56–58). HLA–B*39:06 confers a 10–fold risk of developing the disease, whereas HLA–B*57:01 is highly protective, with an odds ratio of 0.19 (58). HLA class I has also been associated with a risk of progression to type 1 diabetes after seroconversion to diabetes–associated autoantibodies (59) and with the age of onset of type 1 diabetes (60, 61).

**HLA class II and type 1 diabetes**

After extensive research a very high risk associated with having a heterozygous genotype with a haplotype including DRB1*03 on one chromosome and DRB1*04 on the other was reported in the 1980s (62), and this is still known as the most significantly predisposing genotype. Later thousands of investigations are carried out into the association of HLA with type 1 diabetes, including the international Type 1 Diabetes Genetic Consortium (T1DGC), from which the set of global susceptibility genes is largely derived (63–65). The DR–DQ haplotypes conferring the highest risk are DRB1*03:01–DQA1*05:01–DQB1*02:01 (denoted by DR3) and DRB1*04:01/02/04/05/08–DQA1*03:01–DQB1*03:02/04 (or DQB1*02) (denoted by DR4). The heterozygote formed by these gives a 16–fold risk, whereas either of the homozygotes will be associated with a 6–fold risk (66). The highly protective haplotype DRB1*15:01–DQA1*01:02–DQB1*06:02 (denoted by DR2) gives an odds ratio as low as 0.03 (65).

### 2.3.2 Other susceptibility genes

More than 40 genetic loci have been implicated in the risk of type 1 diabetes in genome–wide analyses (32), many of which are related to immune function and are located both inside and outside the HLA region. The association with the disease is far less significant than for the HLA, but these genes have been taken to include the HLA class III region, with somewhat mixed results (67–69). The immune response regulators cytotoxic T–lymphocyte–associated protein 4 (CTLA4) (70), protein tyrosine phosphatase non–receptor type 22 (PTPN22) (71–73), interleukin 2 receptor alpha (IL2RA) and interferon induced with helicase C domain (IFIH1) were among the discoveries to emerge from genome–wide association studies (74). Interestingly, insulin gene polymorphism has been shown to predispose a subject to type 1 diabetes, an effect that may possibly be related to insulin expression and autoimmune target specificity (75, 76).
2.3.3 Genetics in the recognition of future patients

The ultimate goal for research into type 1 diabetes is to find a cure, or possibly even to prevent its onset. Prediction of the appearance of autoantibodies or the onset of the overt disease is particularly important for making prevention possible. Given the relatively low incidence of type 1 diabetes, trials aimed at disease prevention require the screening of a large population in order to find a sufficient number of individuals at risk who will eventually develop the diabetes. Genetic prediction can be used to reduce the target population, of course, making autoantibody screening possible, and it can still capture the majority of future cases (77). The low prevalence of type 1 diabetes nevertheless entails a low positive predictive value, indicating that those who will not develop type 1 diabetes would be at least 10–30 times more numerous than the future cases within the initial population. Prediction is therefore of no use at the individual level (28, 78).

2.4 Environmental factors

The contribution of environmental factors to the pathogenesis of type 1 diabetes has been considered since the 1800s, when case reports of patients with diabetes following chickenpox (79) and mumps infections (80) were published, numerous infectious agents and food items have been studied ever since in order to find a trigger for the autoimmune process.

The fact that the monozygotic concordance rate is approximately 40% demonstrates that environmental factors must modulate genetic susceptibility (81, 82). The geographical variation in the incidence of type 1 diabetes is inconsistent, even among Caucasians, with annual rates of 3.2/100,000 in Macedonia (83) and 64.3/100,000 in Finland in pediatric populations (17). Also the rise in incidence during recent decades, more than doubling since the 1980s, cannot be the consequence only of enhanced genetic susceptibility (17, 84). The few migrant studies available suggest a rise in the incidence of population groups moving from low to high–incidence areas (85, 86), while the proportion of low–risk or even protective HLA genotypes has increased in patients with recent–onset type 1 diabetes (87, 88).
2.4.1 Viral infections

Associations have been suggested between type 1 diabetes and various viral infections, including chickenpox (79), mumps (80), rubella (89), cytomegalovirus (90, 91), Epstein–Barr virus (92), endogenous retroviruses (93) and enteroviruses (94). The strongest evidence has accumulated for enteroviruses. Coxsackie B4 virus was isolated from the pancreas of a child with acute onset diabetes as early as 1979 (95), and only recently a Norwegian study detected a higher prevalence of enteroviral capsid protein and RNA in pancreatic tail resections of living patients with recent–onset type 1 diabetes than in control samples (12). A number of epidemiological studies have given results supporting a role for enteroviral infections in the pathogenesis of diabetes, with a higher prevalence of enterovirus antibodies in patients with recent–onset type 1 diabetes (96, 97). No causal relationship has been proved, however (98, 99), and controversial reports exist even with respect to epidemiological findings (100, 101).

2.4.2 Nutritional factors

Since type 1 diabetes is more common in northern climates, vitamin D levels have been suggested for possible involvement in the pathogenesis of type 1 diabetes, and of other autoimmune disorders (102). It has been observed that vitamin D levels are lower in patients with type 1 diabetes than in the general population (103) and vitamin D supplementation may protect some people from developing the disease (104, 105), but converse results have also been obtained in studies with large samples (106). It is nevertheless the case that both Sardinia and Australia, which have abundant sunlight, have high incidences of type 1 diabetes, which could argue against vitamin D deficiency.

Cow’s milk has been listed as a potential contributor, especially when cross–reactivity with bovine and human insulin was found in the year 2000, although the results were somewhat mixed (107–109), possibly on account of the influence of genetic polymorphism on susceptibility (110). The duration of breastfeeding has also been studied in relation to cow’s milk exposure, again with mixed results (111, 112). In the Trial to Reduce IDDM in the Genetically at Risk (TRIGR), a currently ongoing randomized, double–blinded trial to test whether hydrolyzed infant formula has an effect on the development of type 1 diabetes as compared with a cow’s milk–based formula, no difference was observed between the groups at 7 years (113). The final results will be available after 2017.
Other substances such as omega–3 fatty acids (114), fruit and berry juices (115) and N–nitroso compounds (116) have also been mentioned, but with limited evidence. Further research is needed in this area, too.

2.5 Immune mechanisms in type 1 diabetes

Detectable hyperglycaemia is a late event in the development of the disease, and most of the important immunological events have occurred before the diagnosis. There are many challenges involved in the study of the immunological mechanisms behind type 1 diabetes in humans. 1) The diffuse nature of the endocrine pancreas makes it difficult to examine, especially as the endocrine pancreas forms only 1–2% of the gland volume (117). 2) Inflammation and insulitis seem to be transient and occur only in some of the lobes at any one time (118), and in any case the obtaining of direct biopsies or tissue samples is prone to complications (119, 120). 3) Post–mortem samples are not suitable, as the tissue is prone to rapid autolysis because of the high digestive enzyme content. 4) Improvements in clinical management have resulted in low morbidity with respect to ketoacidosis, leading to a decrease in the number of autopsies from recent–onset type 1 diabetes patients. Taken together, these factors mean that there are few cases available for analysis. In fact the detection of inflammatory infiltration in the islets of Langerhans (insulitis) in humans is based on approximately 150 cases collected during the past 100 years (15).

2.5.1 Insulitis

Lymphocytic infiltration in the islets of Langerhans was described in 1902 in a 10–year–old who died of ketoacidosis (121). The term “insulitis” was first used by the Swiss pathologist von Meyenburg in 1940 (15). At first insulitis was considered to be a relatively rare curiosity, but its true significance was gradually recognized. In 1958 Lecompte (122) observed insulitis to be characteristic of children with acute onset of the disease and short duration, and when a larger cohort of 22 recent–onset cases was studied in 1965 insulitis was observed in 68% of them (123). The transient nature of insulitis was also observed as such islets were no longer present in patients with a disease duration of >1 year (124). The finding that insulitis is present in approximately 20% of islets containing beta cells but was virtually absent in islets from which the beta cells had already disappeared confirmed that the immunological reaction was directed at the beta cells (125, 126).
Quite recently the Juvenile Diabetes Research Foundation (JDRF) – Network for Pancreatic Organ Donors with Diabetes (nPOD) working group (127) defined the diagnostic criterion for classical insulitis as the presence of a predominantly lymphocytic infiltration specifically targeting the islets of Langerhans. The infiltrating cells may be found in the islet periphery (peri–insulitis), or else the infiltrate may be diffuse and present throughout the islet parenchyma (intra–insulitis). The lesion mainly affects islets containing insulin–positive cells and is always accompanied by the presence of (pseudo–) atrophic islets devoid of beta cells. The fraction of infiltrated islets is generally low (<10% of all islet profiles). The lesion should be established in a minimum of three islets with a threshold level of $\geq 15$ CD45+ cells/islet before a diagnosis can be made.

What is currently known of human insulitis in type 1 diabetes is based on the previously mentioned 150 cases (126, 128–130), although a renewed effort to study insulitis using biobanks of human organ donor pancreases has been initiated recently (15, 131).

The lack of new material has led investigators to focus on rodent models for the disease. Biobreeding diabetes–prone (BBDP) rats and the more widely studied non–obese diabetic (NOD) mice (132–134) bear striking similarities to human type 1 diabetes. Both involve a high fraction of T cells, especially of the CD8 type, in addition to macrophages and B lymphocytes in the islets of Langerhans (134), resulting in beta cell destruction at a young age. As in humans, NOD mice show a marked overexpression of HLA class I in their endocrine cells (129, 135, 136), and they also share many other proposed susceptibility genes with humans, including CTLA4, interleukin (IL)–2 and insulin, and display similar humoral responses (137).

2.5.2 Induction of the autoimmune process and cellular immunity

The trigger for inflammation and the autoimmune process is still unknown and only speculations can be made, as already suggested in 1958 (122). For type 1 diabetes to develop in either mice or humans, an immune response to beta cell antigens needs first to be elicited. Second, in order to destroy the beta cells, a proinflammatory response needs to develop. Third, a dysfunction in the mechanisms regulating autoimmune responses is required to allow chronic inflammation and the destruction of beta cells (138).

An infection or other agent may cause the overexpression of proinflammatory cytokines and chemokines in beta cells (139), and this will be followed by the
upregulation of genes associated with the risk of type 1 diabetes, suggesting that
the beta cells themselves contribute to their own demise through positive feedback
to the immune system (139, 140). Also, dysregulation of normally protective gene
transcription processes has been observed (141, 142).

Dendritic cells, which are commonly present even in uninflamed islets both in
humans and mice, take up antigens released from the injured beta cells (143). The
dendritic cells have been shown to be obligatory for the initiation of the anti–islet
immune response, since if they are eliminated no disease will develop (144). The
presentation of antigens occurs via HLA class II molecules on the dendritic cell
surfaces, and as stated before, polymorphism in the HLA complex shows the most
conspicuous association with the risk of type 1 diabetes both in humans and in NOD
mice (32, 121).

Even so, the T cells need to recognize the antigens presented to them for an
autoimmune response to appear. As explained earlier, negative selection in the
thymus is supposed to eradicate self–reactive T cells, but in autoimmune disorders
this selection works inadequately. It seems that both CD4 and CD8 T cells need to
be present for the induction of type 1 diabetes (145).

Both CD8 and CD4 T cells and also CD20 B cells were observed in the islets
of postmortem pancreases (146), while CD8 T cells isolated from recent–onset type
1 diabetes patients with the HLA–A2 (A*0201) allele of the HLA class I complex
recognized and killed human beta cells in vitro (147). It has similarly been found
in NOD mice that diabetes does not occur in the absence of MHC class I and CD8
T cells (148, 149).

CD4 T cells respond to autoantigen peptides that are presented by dendritic
cells with HLA class II molecules (150). In order to trigger an effective CD8 T cell
response to beta cells, it seems that the ability to cross–present exogenous antigens
through HLA class I (which normally presents endogenous peptides) is needed
(151). In order for dendritic cells to acquire this cross–presenting ability, they need
to be stimulated (after HLA class II interaction) through interaction with previously
activated CD4 helper T cells (152). Accordingly, the presence of islet–specific
production of CD4 T cells and their cytokines seems to be crucial for priming an
immune response to the beta cells. In NOD mice the depletion of CD4 T cells leads
to a decrease in the incidence of the disease and can even reverse overt diabetes
(153).

The effects of macrophages and natural killer cells have also been studied, and
these seem to play a role in the development of type 1 diabetes (144, 154–157),
although they are not as crucial as the dendritic, CD4 and CD8 T cells, which seem to be a prerequisite for the onset of the disease (138).

2.5.3 The role of immunoregulation in type 1 diabetes

Control mechanisms exist to keep the effector responses of the immune system on track (158). Immunoregulation is mediated by distinct regulatory populations of cells, either through direct contact or through cytokines. The fact that although most people carry high risk gene variants and many have islet–specific T cells in the blood, only a small proportion of these subjects develop type 1 diabetes (159) refers to success in the regulation of insulitis. Also the penetrance of the disease in NOD mice is not 100%, even though islets are surrounded by cellular infiltrates (160). This indicates that regulation must be interfered with for the disease to develop.

Regulatory T (Treg) cells, characterized by expression of the forkhead box transcription factor (FOXp3), are the most important controllers of immune responsiveness and immunological tolerance in peripheral tissues (161). Their importance is highlighted when loss–of–function mutations in Foxp3 occur, leading to a severe multi–organ autoimmune and inflammatory disorder called IPEX (immune dysfunction, polyendocrinopathy, enteropathy, X–linked inheritance). Along with many autoimmune manifestations, type 1 diabetes breaks out at a young age. A requirement for Tregs in NOD mice to slow the disease down has been demonstrated (162, 163), and the mechanism by which Treg cells suppress immune responses has been subject to extensive research. The production of immunosuppressive cytokines has been described, as also has the killing of antigen–presenting cells by cytotoxic mechanisms (138, 161). A number of studies have presented data indicating a low number of Treg cells or a lowering of their effectiveness (164). Elsewhere suggestions regarding problems of regulation ability have been made (165, 166).

Another species of regulatory cells participating in the pathogenesis of type 1 diabetes is tolerogenic dentritic cells. As mentioned, dendritic cells are essential for the activation of islet–reactive effector T cells (144). Dendritic cells can also modulate immune responses, so that the removal of tolerance–promoting dendritic cells has been found to accelerate the onset of the disease in NOD mice after the pathogenic response has occurred (144). Various ways of inducing a tolerogenic dendritic cell phenotype in vitro with anti–inflammatory agents exist including the use of IL–10, prostaglandin E2, vitamin D and immunosuppressive drugs (167–
These can induce preferential differentiation of Treg cells (171) and also prevent the activation of CD8 T cells (172).

### 2.5.4 Humoral immunity

The role of B cells and beta cell targeting autoantibodies in type 1 diabetes was first described in NOD mice. Mice with deficient B cells through mutation of the immunoglobulin (Ig) gene (173) or through treatment with anti–IgM antibodies (174) developed the disease less often. The presence of islet antigen–specific autoantibodies is highly predisposing factor for diabetes (16, 175), but the autoantibodies themselves are not enough to trigger the disease. Unlike the situation in Grave’s disease, the transfer of maternal autoantibodies through the placenta seems not to influence beta cell function or increase risk of developing type 1 diabetes (176). Similarly, serum that contains autoantibodies does not induce the disease in NOD mice (177, 178). NOD mice with modified B cells that can present antigen but not secrete antibodies have a higher incidence of diabetes than B cell–deficient mice, but still a lower incidence than wild–type NOD mice (178), suggesting that B cells have a role as antigen–presenting cells and that antibodies may also contribute to the pathogenesis by an unknown mechanism.

### 2.5.5 Diabetes–associated autoantibodies

The role of beta cell–specific autoantibodies in the pathogenesis of type 1 diabetes seems to play relatively minor role, as discussed previously, but autoantibodies have been shown to represent an ongoing autoimmune process in the islets of Langerhans and a high predictive value has been achieved for them during past decades by using a combination of autoantibodies directed against beta cell structures. The presence of ≥2 autoantibodies can provide a high predisposition to the onset of type 1 diabetes with a 43.5% risk of progression to the overt disease in 5 years of follow–up and 84.2% in 15 years (16, 179).

**Islet cell antibodies (ICA)**

The presence of ICA reacting with the cytoplasmic structures of endocrine cells within the islets of Langerhans was first reported in a patients with type 1 diabetes and polyendocrine deficiencies in 1974 (180). The presence of ICA has been observed in 60–90% of patients with recent–onset type 1 diabetes (181–183).
Immunofluorescence staining of ICA has shown no precise target antigen, but rather there seem to be a variety of targets, the two major ones being glutamic acid decarboxylase (GAD) and islet antigen 2 (IA–2) proteins (184).

**Insulin autoantibodies (IAA)**

Proinsulin/insulin are the secretory products of pancreatic beta cells and constitute the only beta cell–specific antigen identified so far (185). In 1983 they were found in serum samples from newly diagnosed type 1 diabetes patients before the start of insulin treatment (186).

Since T cell responses to insulin are thought to drive autoreactive B cell responses and autoantibody formation, the search for immunologically relevant regions of the insulin molecule has been focused on T cell epitopes. Amino acids 9–23 in the insulin B chain of NOD mice can lead to the formation of immunogenic epitopes (187), while in human type 1 diabetes T cell responses to insulin have been reported in the peripheral blood (159, 188), pancreatic lymph nodes (189) and the pancreas of a patient with chronic disease (190). Some of the T cells from the pancreas responded to proinsulin epitopes that were restricted to HLA DQ2/DQ8 heterozygous individuals, implying a role for these in the pathogenesis of type 1 diabetes, at least in patients with a fitting genotype (190).

The prevalence of IAA at the time of diagnosis was later shown to be 30–50% and to be highly dependent on age (179, 191). A high titre of IAA at a young age correlates with a rapid course of the disease. IAA are typically associated with an autoimmune process starting at a young age, since individuals with a high IAA titre almost exclusively develop type 1 diabetes before 5 years of age, while less than half of all individuals over 15 years of age at onset carry detectable levels of IAA (192).

**Glutamic acid decarboxylase antibodies (GADA)**

Serum samples obtained from patients with type 1 diabetes were shown in 1982 to immunoprecipitate 64kD proteins from human islets of Langerhans (193), close to 80% of a cohort of recent–onset patients were subsequently shown to have autoantibodies against this 64kD protein (194). In 1992 the protein was identified as the enzyme glutamic acid decarboxylase (GAD) (195), which catalyses the conversion of L–glutamate to γ–amino butyric acid (GABA), the major inhibitory neurotransmitter in the brain (196). Two isoforms of GAD have been identified,
GAD65 and GAD67, encoded by different genes (197). With regard to peripheral tissues, GAD has been detected in the adrenal glands, GI tract, muscles, thyroid gland, thymus, fallopian tubes and testes (198). Only GAD65 is expressed in the islets of Langerhans, where it is localized particularly in the beta cells (199), although its role and function remain unclear (200, 201).

Proliferative T cell responses to GAD65 have been observed in close to 50% of patients with type 1 diabetes and in less than 10% of healthy controls (202), while a loss of tolerance with regard to GAD65 has been suggested as a necessary step in the pathogenesis of type 1 diabetes in NOD mice (203, 204). The T cell response to GAD65 was seen in those studies to occur at the same time as early findings of insulitis, with autoantibodies appearing simultaneously. Accordingly, a GAD vaccination trial was performed using NOD mice, with promising preliminary results (205, 206). This was followed by a human clinical trial in which beta cell insulin secretion was preserved in a sub–group of 11 patients with recent–onset disease (207). Unfortunately, the effects could not be reproduced in later trials (208, 209).

The reported frequencies of GADA in recent–onset type 1 diabetes patients varies between 60 and 85% (210, 211), making this the most common autoantibody observed at the time of diagnosis. An increased frequency of GADA has been observed in individuals carrying the HLA–DQB1*02 allele (210, 212).

Islet antigen 2 antibodies (IA–2A)

The neuroendocrine antigen IA–2 (ICA512) is another major autoantigen associated with the development of type 1 diabetes (213). IA–2 is an enzymatically inactive member of the tyrosine phosphatase family and is involved in regulating insulin secretion. It is a 979–aminoacid protein with a single transmembrane region (214–216), predominantly localized in the secretory granules of neuroendocrine cells (217, 218).

The main immune reactive region of the IA–2 molecule is the intracellular domain of the protein (amino acids 601–979), while other regions have a lesser influence (219). Immune responses to IA–2 have been reported (220), with a proliferative T cell response to IA–2 in 42% of patients with type 1 diabetes and only 8% of healthy controls (220, 221).

The presence of IA–2A at the time of diagnosis was observed in 50–85% of the patients (222–224) and was seen to be associated with the HLA–DR4 (225) and HLA–DQB1*0302 alleles (226).
Zinc transporter family member 8 antibodies (ZnT8A)

ZnT8 is a member of the cation diffusion facilitator family, with high expression in pancreatic beta cells and to a lesser extent in extra–pancreatic tissues (227, 228). In the normal pancreas zinc is concentrated in beta cells and is needed for normal insulin storage. The deletion of ZnT8 from beta cells in mice causes glucose intolerance, inadequate zinc accumulation and dysmorphic insulin granules, as well as a reduced first–phase insulin response after glucose stimulation, a lack of insulin processing enzyme transcripts and an increase in proinsulin levels (229). The relevance of ZnT8 as a prognostic diabetes–associated autoantigen was described by Wenzlau et al. after their evaluation of over 60 candidates obtained by means of a microarray at the mRNA expression level (230). Polymorphism in the intracellular domain of ZnT8 was observed later, and it has been suggested that this could provide broader and more economical screening of patients (231, 232).

ZnT8A are present in 60–80% of recent–onset patients as compared with <2% of healthy controls. Most importantly, they were observed in 26% of subjects with type 1 diabetes who presented with no other commonly used autoantigens of ICA, IAA, GADA or IA–2A. The measurement of ZnT8A has thus successfully provided a more accurate means of screening patients with an ongoing autoimmune process and the basis for a differential diagnosis between type 1 and type 2 diabetes (230, 232). High ZnT8A titres have lately been associated with a more severe disease at onset and with lower C–peptide levels one year after diagnosis (233), although mixed results have been also reported (234).

Other antibodies

As autoimmunity in type 1 diabetes progresses from initial activation to a chronic state of beta cell destruction, there are often higher numbers of autoantigens reacting with T cells, a condition termed “epitope spreading”. In addition to the antibodies mentioned above, a number of others have been found and studied, including ICA 69kD (235, 236), the islet–specific glucose–6–phosphatase catalytic subunit–related protein (237), chromogranin A (238), the antigen jun–B,16, heat shock proteins, the insulin receptor (239), CD38 (240), peripherin (241) and glial fibrillary acidic protein (242) and many others, but so far they have proved to be of minor relevance.
2.5.6 Concluding remarks on immunopathology

The immune mechanisms involved in the mediation of beta cell destruction have been actively investigated for decades. Based on experimental observations, ways of curing type 1 diabetes or slowing down the disease process through the depletion of adaptive immune cells (243–247), the dampening of innate immune responses with a cytokine blockade (248–250), the induction of antigen–specific tolerance (207, 208, 251–253) and the boosting of Treg cells have been tested (254). Often preclinical studies have shown promising effects but the subsequent trials have been disappointing (Table 3). None of the approaches has so far succeeded in restoring the long–lasting effect of endogenic insulin secretion (254). Whether the lack of success in translating the efficacy observed in animal models to the clinic is connected with problems of timing or the dose of the intervention agent, or whether it represents differences between the species is still not known.

The heterogeneity in the clinical profiles of patients may also mean that, even if the therapeutic agent proves a success in a minority of individuals, the result may still remain invisible due to the inhomogeneity of the population studied. Research aimed at achieving a greater understanding of the immunomechanisms behind beta cell death, repair and regeneration should probably be integrated with approaches to finding a therapeutic strategy for handling type 1 diabetes. A simplified diagram of the pathogenesis of type 1 diabetes is presented in Fig. 2.
Fig. 2. Simplified schematic diagram of the pathogenesis of type 1 diabetes. Dendritic cells, HLA class I, CD4 and CD8 T cells seem to be prerequisites for the development of the disease.

2.6 Glucose metabolism in type 1 diabetes and the preclinical period

According to the World Health Organization (WHO) and the American Diabetes Association (ADA) (255), the diagnosis of type 1 diabetes is based on fasting plasma glucose ≥7 mmol/l, any blood glucose reading of ≥11.1 mmol/l with symptoms of hyperglycaemia, or diagnostic plasma glucose values in two separate oral glucose tolerance tests (OGTTs) in an asymptomatic patient. In 2009 the ADA included HbA1c ≥6.5% (48 mmol/mol) as a diagnostic criterion for type 2 diabetes (255, 256).

Type 1 diabetes was without exception a lethal disease before the discovery of insulin in 1921–1922, and even with the availability of exogenous insulin its metabolic regulation can be inadequate and associated complications are common.
(e.g. retinopathy, neuropathy, cardiovascular disease). Patients have traditionally injected insulin in accordance with self–monitored blood glucose (SMBG) measurements obtained with a glucometer, but nowadays diabetes management has evolved to emulate the physiological role of the endocrine pancreas by means of a number of mechanical technologies such as insulin pumps and continuous glucose monitoring (CGM) (257).

The use of continuous subcutaneous insulin infusions with insulin pumps has increased in recent times (257), and randomized controlled trials have shown that lower HbA1c concentrations can be achieved with sensor–augmented pump therapy than with injection therapy (258, 259). A CGM system employing modern technology was established in 2006 that included a sensor inserted subcutaneously sending real–time information to a monitoring device. The use of this device was found to correlate with decreased lengths of time spent in hypoglycaemia (<4 mmol/l) and with lowered HbA1c levels (260). Also, less nocturnal hypoglycaemia was found to occur, this being particularly important for pediatric patients and their families (261). Currently the two technologies are often combined into a form of sensor–augmented pump therapy, with good results (258, 262).

Although the monitoring of glucose is a routine aspect of the care provided for chronic type 1 diabetes, at least in the developed countries, less is known about glucose metabolism prior to diagnosis and the development of glucose intolerance. Our knowledge is based on follow–up studies of diabetes–associated antibodies from birth in individuals possessing genetic risk factors, as in the Type 1 Diabetes Prediction and Prevention (DIPP) project, the Trial to Reduce IDDM in the Genetically at Risk (TRIGR), the Environmental Determinants of Diabetes in the Young (TEDDY) study and the Diabetes and Autoimmunity Study in the Young (DAISY), or on examinations of the relatives of patients with type 1 diabetes, as in the Diabetes Prevention Trial–Type 1 (DPT–1) and the TrialNet Natural History study (179, 263–265). Glucose parameters have been recorded for years, finally resulting in an enhanced understanding of how glucose metabolism behaves in high–risk children. It is still the case, however, that only limited amounts of data are available on individuals with no signs of autoimmunity.

The methods used to describe glucose metabolism in follow–up studies have been the traditional measurements of plasma glucose, HbA1c and OGTT and the intravenous glucose tolerance test (IVGTT).

The only assessment of plasma glucose without any special instructions as to what constitutes an adequate sample size is from the Finnish DIPP Study, which showed a rise in plasma glucose 1.5 years before diagnosis in those autoantibody–
positive children who later developed type 1 diabetes as compared with autoantibody positive controls who did not develop diabetes during the follow–up (II).

HbA1c has been used less often in follow–up studies. The first observation of a rise in HbA1c before diagnosis was recorded in 2006 in a small study population of 28 children who progressed to type 1 diabetes (266).

OGTT is the gold standard for diagnosing diabetes (267). Abnormal values for either fasting plasma glucose or 2–hour glucose have frequently been observed, especially from 6 months to 1.5 years before diagnosis (268). OGTT samples taken at time points of 0, 30, 60, 90, 120 minutes, including glucose and C–peptide levels, have revealed a rise in glucose values and a corresponding decline in C–peptide at least 6 months before diagnosis (269).

IVGTT illustrates the capacity of the beta cells to secrete insulin after infusion of a glucose dose of 0.5g/kg (maximum 35g). The first 10 minutes represent the acute insulin response to a rapid glucose stimulus (270), and the initial burst occurs via a release of insulin from the already prepared secretory granules within the beta cells (271). The often used first–phase insulin response (FPIR) is calculated from the sum of serum insulin concentrations at 1 and 3 minutes. The decline in this value in individuals who progressed to type 1 diabetes relative to high–risk autoantibody–positive controls was found to occur approximately 1.5 years before diagnosis (272, 273), and the FPIR values of the progressor group were lower than those of low–risk controls with no biochemical autoantibodies (IAA, GADA, IA–2A or ZnT8A) as early as 4–6 years before diagnosis (274). This has brought up the possibility of an intrinsic defect in beta cell mass or function, which nevertheless remains to be confirmed. The suggestion is also supported by findings of an abnormally small pancreas in patients with type 1 diabetes and the more frequent occurrence of irregular vascularization (7).

2.7 Assessment of the risk of progression to type 1 diabetes

The pathogenesis of type 1 diabetes can be categorized according to inherited risk factors and detectable biomarkers into three preclinical stages (275). Before preclinical diabetes, individuals may possess a predisposing genetic risk, mostly influenced by their HLA class II genotype, but also by many other factors with smaller effects (78).

Stage 1 represents individuals who have developed ≥2 biochemical autoantibodies (IAA, GADA, IA–2A or ZnT8A) but are normoglycaemic.
Progression to overt disease is almost inevitable for these children, with a 84% risk of occurring during a 15–year follow–up (16).

Stage 2 includes individuals with ≥2 autoantibodies but whose disease has progressed to a point where glucose intolerance or dysglycaemia can be detected due to insufficient beta cell mass. At this stage the 5–year risk is over 80% (276).

By stage 3 typical symptoms of type 1 diabetes have appeared, including polyuria, polydipsia, polyphagia, weight loss and fatigue, together with diagnostic values for glucose parameters (175, 255).

2.7.1 Genetic susceptibility and family history

The HLA region accounts for about 50% of the genetic risk of developing type 1 diabetes, the most significant haplotypes being DR3–DQ2 and DR4–DQ8 (33), although the rising incidence of type 1 diabetes has been accompanied by a fall in the relative contributions of the highest HLA risk genotypes (17, 277). The rest of the currently known genetic risk can be attributed to approximately 40 non–HLA loci identified mostly via genome–wide association studies (78). The highest risks among non–HLA genes are associated with the INS, PTPN22, CTLA4 and IL2RA genes (278). HLA risk genes have so far been used mostly in follow–up studies, as inclusion criteria for achieving smaller sample sizes in the DIPP, TRIGR, TEDDY and DAISY projects (179, 263–265). The frequencies of the genotypes among patients with diabetes have been well characterized in Finland (56, 279).

The impact of genotype can be observed in relatives of individuals with type 1 diabetes, who have increased risk compared to the general population (280, 281). Children in the highest risk group, those having an identical twin with type 1 diabetes, several affected first–degree relatives or a first–degree relative with type 1 diabetes, might benefit from HLA screening (280–284). If such an individual possesses a high–risk genotype the risk of developing type 1 diabetes will be over 10% and frequent examinations for the appearance of suspicious symptoms has been considered useful (285). Convincing evidence of less severe clinical status at disease onset has been presented in the case of children with an affected first–degree and second–degree relative (28, 286, 287), but whether this is due to the earlier detection of typical symptoms remains unknown.

This increased risk in relatives of individuals with type 1 diabetes has been used for research purposes in the follow–up aspects of the DPT–1 (288, 289) and TrialNet Natural History studies (290).
It should be emphasized that the majority of individuals with an HLA risk never develop symptomatic type 1 diabetes, and therefore the positive predictive value of genotype testing is low (Table 1).
Table 1. Type 1 diabetes and genetic susceptibility in Caucasian population. The HLA risk alleles included in the Table are HLA DRB1*03 and *04 and DQB1*0302 and the HLA protective alleles are HLA DQB1*0602, *0301, *0303, *0603 and *0503 (275).

<table>
<thead>
<tr>
<th>Population</th>
<th>Risk of type 1 diabetes, %</th>
<th>Frequency in population, %</th>
<th>Frequency in T1D patients, %</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low risk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian newborns</td>
<td>0.4–1</td>
<td>100</td>
<td>100</td>
<td>17</td>
</tr>
<tr>
<td>Protective genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FDRs of T1D patients with</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>protective genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate risk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Risk genotype</td>
<td>4</td>
<td>4–5</td>
<td>36</td>
<td>291</td>
</tr>
<tr>
<td>Risk genotype of HLA and non–</td>
<td>12</td>
<td>1</td>
<td>27</td>
<td>292</td>
</tr>
<tr>
<td>HLA genes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FDRs of T1D patients</td>
<td>5</td>
<td>0.5–1</td>
<td>10</td>
<td>282</td>
</tr>
<tr>
<td>High risk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FDRs of T1D patients with high</td>
<td>10–20</td>
<td>0.1</td>
<td>0.05</td>
<td>282</td>
</tr>
<tr>
<td>risk genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FDRs of T1D patients with high</td>
<td>40</td>
<td>0.1</td>
<td>0.05</td>
<td>292</td>
</tr>
<tr>
<td>risk HLA and non–HLA genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥2 affected first–degree relatives</td>
<td>20–25</td>
<td>0.001</td>
<td>0.005</td>
<td>283</td>
</tr>
<tr>
<td>Very high risk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Identical twin with type 1 diabetes</td>
<td>30–70</td>
<td>0.001</td>
<td>0.005</td>
<td>280, 281</td>
</tr>
</tbody>
</table>

first–degree relative (FDR), type 1 diabetes (T1D)

2.7.2 Humoral risk factors

The presence of ≥2 islet autoantibodies, which can be defined as Stage 1 preclinical type 1 diabetes (275), was detected as early as 6 months of age in the Finnish DIPP
study and peaked between 9 and 24 months of age, at a median age of 15 months (293). A similar observation was made in the TEDDY study (294). The have been a number of reports of the appearance of specific autoantibodies associated with age (293, 295) and HLA genotype (296–298), and the speed of the progression from seroconversion to symptomatic disease has also been shown to depend on age at seroconversion, the magnitude of the autoimmunity titre and the appearance of IA–2A as the first autoantibody (216, 295, 299). Even so, screening for islet autoantibodies has demonstrated that, regardless of other factors, children with ≥2 positive autoantibodies have a lifetime risk of type 1 diabetes that approaches 100% (16).

2.7.3 Metabolic risk factors

Stage 2 prediabetes includes individuals with ≥2 autoantibodies and detectable glucose intolerance or dysglycaemia. A number of definitions have been put forward for this (Table 2): FPIR in IVGTT (272, 274, 300), impaired fasting plasma glucose (IFG; ≥6.1 mmol/l), impaired glucose tolerance in OGTT (IGT; ≥7.8 mmol/l) and high plasma glucose levels at varying points in time during OGTT (301–306), C–peptide levels (269, 276, 301), random plasma glucose (II), a rise in HbA1c (266) or possibly CGM results (307–309). At this stage in the disease the risk is approximately 50% during the first year, 60–70% in 2 years and roughly 80% in 5 years, depending on the test used, with high positive predictive value (268, 276). Individuals at stage 2 can be recognized with very good specificity, but again with somewhat poorer sensitivity. At stage 3 diagnosis is usually easy, as the typical symptoms appear and it is only the distinction between type 1, type 2 and rarer types that can be difficult (310).
Table 2. Significant predictive glucose parameters obtained in follow–up studies of children with either first–degree relatives with type 1 diabetes and positive autoantibodies, or HLA risk detected at birth and later seroconversion to autoantibodies.

<table>
<thead>
<tr>
<th>Study</th>
<th>Test</th>
<th>Suggested predictive parameter</th>
<th>Time point of observed difference</th>
<th>Risk of type 1 diabetes after detection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIPP</td>
<td>Plasma glucose</td>
<td>≥7.8 mmol/l</td>
<td>1.5 years before diagnosis</td>
<td>Median time 1.1 years, HR 6.0</td>
<td>II</td>
</tr>
<tr>
<td>DIPP</td>
<td>HbA1c</td>
<td>10% rise between 3–12 months or ≥5.9% twice</td>
<td>2.0 years before diagnosis</td>
<td>Median time 1.1 and 0.9 years, HR 5.7 and 11.9</td>
<td>I</td>
</tr>
<tr>
<td>TrialNet</td>
<td>HbA1c</td>
<td>10% rise from baseline</td>
<td>NA</td>
<td>2–year incidence 20%</td>
<td>276</td>
</tr>
<tr>
<td>DPT–1, TEDDY, TRIGR, TrialNet</td>
<td>HbA1c</td>
<td>HbA1c ≥5.7%</td>
<td>Observed ability to recognize dysglycaemia detected in OGTT</td>
<td>Sensitivity 26.4%, specificity 85.7%</td>
<td>311</td>
</tr>
<tr>
<td>DIPP</td>
<td>OGTT</td>
<td>IFG</td>
<td>At diagnosis</td>
<td>HR 3.2</td>
<td>II</td>
</tr>
<tr>
<td>DIPP</td>
<td>OGTT</td>
<td>IGT</td>
<td>1.5 years before diagnosis</td>
<td>HR 8.3</td>
<td>II</td>
</tr>
<tr>
<td>TrialNet</td>
<td>OGTT</td>
<td>Abnormal value at any point in OGTT</td>
<td>NA</td>
<td>2–year incidence 41%</td>
<td>276</td>
</tr>
<tr>
<td>DPT–1</td>
<td>OGTT</td>
<td>2–hour glucose &gt;6.33 mmol/l</td>
<td>NA</td>
<td>NA</td>
<td>302</td>
</tr>
<tr>
<td>TrialNet, DPT–1</td>
<td>C–peptide in OGTT</td>
<td>≥20% and ≥30% decrease in AUC from baseline</td>
<td>6 months before diagnosis</td>
<td>2–year incidence 39% and 20%</td>
<td>269, 276</td>
</tr>
<tr>
<td>DPT–1</td>
<td>C–peptide in OGTT</td>
<td>Difference of 0– and 30 min values</td>
<td>2.0 years before diagnosis</td>
<td>NA</td>
<td>312</td>
</tr>
<tr>
<td>DPT–1</td>
<td>C–peptide in OGTT</td>
<td>Slowly rising C–peptide</td>
<td>2.0 years before diagnosis</td>
<td>NA</td>
<td>312</td>
</tr>
<tr>
<td>DPT–1</td>
<td>C–peptide in OGTT</td>
<td>Peak C–peptide</td>
<td>NA</td>
<td>NA</td>
<td>302</td>
</tr>
<tr>
<td>Study</td>
<td>Test</td>
<td>Suggested predictive parameter</td>
<td>Time point of observed difference</td>
<td>Risk of type 1 diabetes after detection</td>
<td>Reference</td>
</tr>
<tr>
<td>---------</td>
<td>--------</td>
<td>---------------------------------</td>
<td>-----------------------------------</td>
<td>----------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DIPP</td>
<td>IVGTT</td>
<td>FPIR</td>
<td>4 to 6 years before diagnosis</td>
<td>NA</td>
<td>274, 313</td>
</tr>
<tr>
<td>DPT–1</td>
<td>IVGTT</td>
<td>Fasting insulin 10.23 mU/l</td>
<td>NA</td>
<td>NA</td>
<td>302</td>
</tr>
<tr>
<td>DPT–1</td>
<td>IVGTT</td>
<td>FPIR 156 mU/l</td>
<td>NA</td>
<td>NA</td>
<td>302</td>
</tr>
<tr>
<td>DPT–1</td>
<td>IVGTT</td>
<td>50% decline in FPIR</td>
<td>0.5 to 1.5 years before diagnosis</td>
<td>NA</td>
<td>272</td>
</tr>
<tr>
<td>DPT–1</td>
<td>IVGTT</td>
<td>FPIR below first percentile of control values</td>
<td>NA</td>
<td>Incidence 0.48/year compared with 0.05/year in controls</td>
<td>300</td>
</tr>
<tr>
<td>DPT–1</td>
<td>IVGTT</td>
<td>HOMA–IR</td>
<td>NA</td>
<td>HR 2.7</td>
<td>314</td>
</tr>
<tr>
<td>Melbourne</td>
<td>IVGTT</td>
<td>HOMA–IR</td>
<td>NA</td>
<td>HR 1.65</td>
<td>315</td>
</tr>
<tr>
<td>DIPP</td>
<td>CGM</td>
<td>Time spent ≥7.8 mmol/l</td>
<td>NA</td>
<td>NA</td>
<td>III</td>
</tr>
<tr>
<td>DAISY</td>
<td>CGM</td>
<td>Time spent ≥7.8 mmol/l</td>
<td>NA</td>
<td>NA</td>
<td>308</td>
</tr>
<tr>
<td>Brussels</td>
<td>CGM</td>
<td>Increased variability</td>
<td>NA</td>
<td>80% sensitivity and specificity</td>
<td>309</td>
</tr>
</tbody>
</table>

hazard ratio (HR), impaired fasting glucose (IFG), impaired glucose tolerance (IGT), first-phase insulin response (FPIR), continuous glucose monitoring (CGM), homeostatic model assessment of insulin resistance (HOMA–IR)
Overall, it can be stated that the risk of progression to type 1 diabetes can be successfully predicted if genetic, humoral and metabolic screening is performed, as long as intervention to preserve beta cell function or even prevent type 1 diabetes remains impossible there are only minor benefits to be gained from this, so that screening of the general population is not recommended (275, 316). The ability to predict and properly stage prediabetes may be useful for research purposes, however, as it can facilitate the designing of smarter, shorter and less expensive clinical trials using eligible subjects (276). Our knowledge of the predictable aspects of progression is already being used in clinical trials (275), although the wide variation in the time from seroconversion to diagnosis and the age at onset, and probably individual factors such as the original beta cell mass and perhaps the intensity of the autoimmune reaction, will continue to make prediction difficult.

The ethics of the follow-up studies have been questioned since currently there are no preventative treatments available (317). However, it is also noteworthy that identification of emerging type 1 diabetes may help the families to prepare for the lifelong treatment, and earlier diagnosis of type 1 diabetes has been shown to prevent diabetic ketoacidosis at disease onset and shorten the initial hospitalization (318).
2.8 Evidence for different subtypes of type 1 diabetes

Recent studies of the histology of the human pancreas have added support to the concept of the existence of different subtypes of type 1 diabetes. Histological studies of patients with disease onset at age 0–14 years and samples obtained less than one month after diagnosis show over 70% inflamed islets compared to 30% found in older patients (15), suggesting a more aggressive disease process. Also, high levels of CD20 B cells, CD45 cells and CD8 T cells have been observed in the pancreases of younger patients (136, 319, 320).

Inflammation is also present in the pancreatic exocrine tissue of some patients, as shown in nPOD studies (321, 322). Regarding the aetiology of this, it has been speculated that it could the result of genes that cause tissue–based inflammation or of a predisposition to infectious agents such as viruses (323, 324). Coxsackievirus capsid protein and viral RNA have recently been found in islets (12, 13, 325). Inadequate immune mediation in individuals with susceptibility genes or increased numbers of dendritic cells producing cytokines that modulate antiviral responses could cause persistent viral infection in the islets of certain individuals (326–329).

The normal volume of the pancreas has been considered to be around 90 cm³, with the islets accounting for approximately 1–2% of this. Studies of cadaveric pancreases have shown, however, that beta cell mass varies up to five–fold in healthy individuals independent of age or BMI (330–332). The fact that pancreatic mass is frequently smaller in patients with recent–onset diabetes than in healthy controls may suggest deficiencies in organogenesis, possibly predisposing the pancreas to undesirable immunoreactions (333, 334).

In the commonly accepted historical model the autoimmune process continues until all the beta cells are lost, but the reality again seems to be different. Many individuals with chronic type 1 diabetes produce small amounts of C–peptide and histological studies of these pancreases have shown the presence of insulin–positive beta cells either in islets or scattered around the exocrine pancreas (320, 335, 336). These C–peptide levels have been higher in patients with disease onset at >18 years of age (335). The question why some of the beta cells in certain individuals survive the autoimmune attack remains to be answered, but suggestions have included the possibility of more effective beta cell regeneration (336, 337).
2.9 Prevention of type 1 diabetes

The first randomized, double–blinded trials with sufficient statistical power that aimed at preventing or reversing type 1 diabetes were conducted in the mid–1980s using cyclosporine (338–340). In both trials remission was observed in the groups that received cyclosporine as compared with a placebo, but the duration of the benefit was short and did not justify the inevitable adverse effects. These efforts did awake a wide interest in immunotherapy as a potential cure for type 1 diabetes, however.

Numerous clinical trials have been conducted since the two cyclosporine studies, using varying immunological strategies (Table 3), but mostly the results have been disappointing, with none of the agents producing any clinically relevant effect on beta cell function.
Table 3. Intervention trials focused on type 1 diabetes. A primary prevention study means that the intervention commenced was before the appearance of diabetes–associated autoantibodies, while a secondary prevention study started after seroconversion to autoantibodies but before any clinical symptoms were present. Depending on the study, the outcome was defined as either the diagnosis of type 1 diabetes, the appearance of autoantibodies, an effect on C–peptide levels or a need for insulin. In addition to those listed below, there are many studies still in progress (338).

<table>
<thead>
<tr>
<th>Study</th>
<th>Intervention</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary prevention</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRIGR pilot</td>
<td>Hydrolyzed casein formula</td>
<td>Benefit</td>
<td>341</td>
</tr>
<tr>
<td>TRIGR*</td>
<td>Casein hydrolysate formula</td>
<td>No difference</td>
<td>113</td>
</tr>
<tr>
<td>BABYDIET</td>
<td>Gluten–free diet</td>
<td>No difference</td>
<td>342</td>
</tr>
<tr>
<td>FINDIA</td>
<td>Insulin–free whey–based formula</td>
<td>Benefit</td>
<td>343</td>
</tr>
<tr>
<td><strong>Secondary prevention</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DENIS</td>
<td>Nicotinamide</td>
<td>No difference</td>
<td>344</td>
</tr>
<tr>
<td>ENDIT</td>
<td>Nicotinamide</td>
<td>No difference</td>
<td>345</td>
</tr>
<tr>
<td>DPT–1 Parenteral insulin</td>
<td>Injected insulin</td>
<td>No difference</td>
<td>288</td>
</tr>
<tr>
<td>DPT–1 Oral Insulin</td>
<td>Oral insulin</td>
<td>No difference</td>
<td>252</td>
</tr>
<tr>
<td>DIPP birth cohort</td>
<td>Nasal insulin</td>
<td>No difference</td>
<td>251</td>
</tr>
<tr>
<td>DIPP sibling cohort</td>
<td>Nasal insulin</td>
<td>No difference</td>
<td>251</td>
</tr>
<tr>
<td>Belgian parenteral insulin</td>
<td>Injected insulin</td>
<td>No difference</td>
<td>346</td>
</tr>
<tr>
<td><strong>Recent–onset diabetes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>French cyclosporine</td>
<td>Cyclosporine</td>
<td>Benefit</td>
<td>339</td>
</tr>
<tr>
<td>Canadian–European cyclosporine</td>
<td>Cyclosporine</td>
<td>Benefit</td>
<td>340</td>
</tr>
<tr>
<td>Azathioprine, adults</td>
<td>Azathioprine</td>
<td>Benefit</td>
<td>347</td>
</tr>
<tr>
<td>Azathioprine, children</td>
<td>Azathioprine</td>
<td>Benefit</td>
<td>348</td>
</tr>
<tr>
<td>Azathioprine</td>
<td>Azathioprine and prednisone</td>
<td>Benefit</td>
<td>349</td>
</tr>
<tr>
<td>Linomide French trial</td>
<td>Linomide</td>
<td>Benefit</td>
<td>350</td>
</tr>
<tr>
<td>BCG</td>
<td>BCG vaccine</td>
<td>No difference</td>
<td>351, 352</td>
</tr>
<tr>
<td>French oral insulin</td>
<td>Oral insulin</td>
<td>No difference</td>
<td>353</td>
</tr>
<tr>
<td>Italian oral insulin</td>
<td>Oral insulin</td>
<td>No difference</td>
<td>354</td>
</tr>
<tr>
<td>Anti–CD3 trials</td>
<td>Teplizumab</td>
<td>Mostly benefit</td>
<td>243, 244, 355–358</td>
</tr>
<tr>
<td>Anti–CD3 trials</td>
<td>Otelixizumab</td>
<td>Mostly no difference</td>
<td>247, 359–361</td>
</tr>
<tr>
<td>GAD–vaccine trials</td>
<td>GAD–alum vaccine</td>
<td>No difference</td>
<td>207–209</td>
</tr>
</tbody>
</table>
A number of misleading pilot studies have demonstrated interventions that have been successful in delaying the onset of the disease. Increasing the immunotolerance with low-dose insulin was successful in NOD mice and in two pilot studies with humans (385, 386), but a randomized controlled clinical trial with a cohort of 339 autoantibody-positive patients enrolled after the screening of a total of 93,000 individuals led to the surprising outcome that there was absolutely no effect. A trial using GAD–alum vaccines was also performed after successful pilot studies, but again with no benefit. Other schemes that were promising at the pilot stage but ended up with disappointments in full-powered clinical trials concerned DiaPep277 (derived from human heat shock protein 60) and Bacillus Calmette–Guerin (BCG) vaccine (Table 3).

Pilot studies have proved to be disadvantageous in this area and should be viewed with great caution. Definite answers can be achieved only by means of adequately powered randomized controlled trials. Pilot studies do offer a glimpse

<table>
<thead>
<tr>
<th>Study</th>
<th>Intervention</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DiaPep277 peptide trials</td>
<td>DiaPep277 peptide</td>
<td>At first benefit, later no difference</td>
<td>362–368</td>
</tr>
<tr>
<td>MMF/DZB</td>
<td>Mycophenolate mofetil with/without daclizumab</td>
<td>No difference</td>
<td>369</td>
</tr>
<tr>
<td>Anti–CD20 TrialNet</td>
<td>Anti–CD20 rituximab</td>
<td>Benefit</td>
<td>245, 370</td>
</tr>
<tr>
<td>Abatacept TrialNet</td>
<td>Abatacept</td>
<td>Benefit</td>
<td>371, 372</td>
</tr>
<tr>
<td>Canakinumab TrialNet</td>
<td>Anti–IL1b canakinumab</td>
<td>No difference</td>
<td>250</td>
</tr>
<tr>
<td>ITN</td>
<td>Thymoglobulin</td>
<td>No difference</td>
<td>373</td>
</tr>
<tr>
<td>T1DAL–alefacept ITN</td>
<td>Alefacept</td>
<td>No difference</td>
<td>374</td>
</tr>
<tr>
<td>IL–2 &amp; rapamycin safety ITN</td>
<td>IL–2 and rapamycin</td>
<td>Harmful</td>
<td>375</td>
</tr>
<tr>
<td>AIDA anakinra trial</td>
<td>Anakinra</td>
<td>No difference</td>
<td>250</td>
</tr>
<tr>
<td>Altered peptide ligand</td>
<td>B9–23 altered peptide ligand</td>
<td>No difference</td>
<td>376</td>
</tr>
<tr>
<td>ATG–GCSF trial</td>
<td>ATG and GCSF</td>
<td>Benefit</td>
<td>377</td>
</tr>
<tr>
<td>DIATOR</td>
<td>Atorvastatin</td>
<td>No difference</td>
<td>378</td>
</tr>
<tr>
<td>REPAIR–T1D</td>
<td>Sitagliptin and lansoprazole</td>
<td>No difference</td>
<td>379</td>
</tr>
<tr>
<td>AHScT + profound immunosuppression</td>
<td>Cyclophosphamide, GCSF, ATG, AHSCT</td>
<td>Benefit</td>
<td>380–383</td>
</tr>
<tr>
<td>Low–dose IL–2 safety trial</td>
<td>IL–2 (3 doses)</td>
<td>Increased Treg number</td>
<td>384</td>
</tr>
</tbody>
</table>

*The final results of the TRIGR study will be achieved in 2017.*
of hope, however, for it is tempting to think that the initial finding might perhaps be lost in larger studies because the patient material is too heterogeneous. If we were to learn to phenotype type 1 diabetes more accurately, by distinguishing subtypes of autoimmune reactions, for example, could we then elicit a solution?

Ideal future immunotherapies would be ones that are specific to type 1 diabetes and entail minimal adverse effects. Antigen–based tolerance–inducing therapies would be fit the bill, and, as discussed earlier, these have been very successful with animal models (387, 388) but have had no success with humans (Table 3). Possible explanations for this have been proposed. Since there is a growing evidence for different subtypes lying behind the autoimmune process, there have been suggestions that “responders” should be separated from the other patients in trials. For example, in one trial using the anti–CD3 monoclonal antibody teplizumab a group of responders had maintained C–peptide levels at 2 years by comparison with the control group (358). A total of 48% of the individuals treated with the drug had a positive response, but this was lost if the treatment group was analysed as a whole. If a “responder analysis” of this kind could be included in all intervention trials concerned with type 1 diabetes we could maybe learn more about the differentiation of phenotypes (389).

The question remains as to why some subjects respond to treatment and others do not. Many explanations can be proposed: an ineffective type of immunotherapy, the wrong dosage, major existing beta cell damage, differences in beta cells between subjects, or maybe the pulsating nature of insulitis, which would require administration of the drug to be intermittent (338).

It should be accepted that several immune pathways are certainly implicated in the evolution of type 1 diabetes, as in other autoimmune diseases, and this may explain why treatment with a single agent has yielded no permanent results. We are now hoping for combinations of therapies that might exceed the threshold level for clinically meaningful effects, but while progress has clearly been made towards an understanding of the pathogenesis of type 1 diabetes, we can conclude, as usual, that many more investigations and discoveries will be needed before any answers are forthcoming.
3 Aims of the research

Islet autoantibodies have significantly improved the ability to predict the risk of type 1 diabetes. Still, there has been a lack of a proper way to predict the timing of the onset. The objectives of the present work were:

1. to characterize the HbA1c values during preclinical type 1 diabetes and predict the time to diagnosis with HbA1c in children with a high risk of developing type 1 diabetes,
2. to compare the predictive characteristics of OGTT and random plasma glucose in high-risk children, and
3. to investigate differences in daily glucose values obtained with CGM between children with a high or low risk of developing type 1 diabetes.
4 Materials and methods

4.1 Study design

The population included in this work consisted of participants in the DIPP study, a Finnish population–based study in which children with HLA–conferring susceptibility to type 1 diabetes are observed prospectively from birth. Recruitment of newborn infants started in the university hospitals of Oulu, Tampere and Turku in Finland in November 1994 and is still continuing. Screening for genetic risk is performed from cord blood, and all the families with an infant carrying HLA genotypes associated with type 1 diabetes are invited to prospective follow–up examinations at 3 to 12–month intervals until the age of 15 years or until the clinical disease is diagnosed. Islet autoantibodies are analysed at each visit. If even a single positive autoantibody is detected the visits are scheduled to take place every three months. If seroconversion to ≥2 autoantibodies occurs, the monitoring of glucose metabolism is started. HbA1c is measured at every visit, random plasma glucose twice a year, and OGTT and IVGTT are performed once a year. The diagnosis of type 1 diabetes is based on typical symptoms and random high plasma glucose levels, or diagnostic plasma glucose values in two separate oral glucose tolerance tests in the case of an asymptomatic patient, as suggested by the WHO (267).

The population for the current work (I, II) comprised 567 autoantibody–positive children (Fig. 3), of whom 255 developed type 1 diabetes and 312 remained disease–free until the end of the follow–up on December 31st 2011. Since not all the glucose parameters were obtained for every participant, the final numbers of children were 466 (201 progressors, 265 non–progressors) in the HbA1c analysis, 403 (209 progressors, 194 non–progressors) in OGTT and 505 (204 progressors, 301 non–progressors) in the random plasma glucose series.

The population in the CGM study (III) comprised of 10 high–risk children with ≥2 positive autoantibodies and 10 age and sex–matched low–risk controls with HLA susceptibility but no signs of autoimmunity during the follow–up. All the children were recruited from the DIPP study. The CGM readings were not visible during the study itself, but the participants calibrated the device with a SMBG reading twice a day, with no specific instructions related to meals. All the children were invited to for a 5–point OGTT in which plasma glucose and C–peptide values were analysed at 0, 30, 60, 90 and 120 minutes. HbA1c sample was also taken. The
families kept food records throughout the follow–up week. Amount and time of consumed carbohydrates were analyzed together with CGM results.

The study was approved by the Ethics Committees of the participating university hospital districts. All the participant families provided written informed consent.
4.2 Genetic screening

HLA–conferred susceptibility to type 1 diabetes was analysed centrally using cord blood. The major Caucasian HLA–DR/DQ haplotypes were defined using panels of sequence–specific oligonucleotide probes as described in previous publications from the DIPP study (390). Children with increased genetic risk were invited for regular follow–up examinations.
4.3 Immunological screening

ICA was measured using indirect immunofluorescence. Antibodies against insulin, GAD65, and IA–2 were analysed with specific radiobinding assays using cut–off rates based on the 99th percentile for >350 non–diabetic subjects (179). The sensitivities and specificities of the assays in the DIPP laboratory according to the 2002 and 2010 Diabetes Antibody Standardization Programme workshops were 44–50% and 96–99% for IAA, 82–92% and 94–97% for GADA, and 64–72% and 97–100% for IA–2A, respectively. The primary islet autoantibodies analysed in paper II were defined as the first autoantibodies detected, or the first combination if multiple islet autoantibodies occurred in the same sample.

4.4 Metabolic assays

4.4.1 HbA1c

The immunoassay–based method for measuring HbA1c was employed throughout the study period at Oulu University Hospital, while Fast Protein Liquid Chromatography (FPLC) was used at Tampere University Hospital until 9th June 1999 and the immunoassay–based method after that. At Turku University Hospital the FPLC method was used until 5th August 1996 and the High Performance Liquid Chromatography (HPLC) method after that.

4.4.2 Oral glucose tolerance test

Oral glucose (1.75 g/kg body weight, up to a maximum of 75 g) was administered after overnight fasting. Samples were taken at 0 and 120 min and tested for glucose in the local laboratory. Capillary samples were used at Oulu University Hospital, whereas in Tampere and Turku the tests were based on venous samples. A glucose dehydrogenase–based method was used in Oulu until May 2000 and a glucose oxidase method thereafter, while in Tampere and Turku the hexokinase method was employed throughout. Statistical analyses of these methodological differences are presented in detail in the relevant sections of the original papers.
**4.4.3 Random plasma glucose**

Random plasma glucose samples were obtained from venous plasma in all three hospitals. In Oulu the glucose dehydrogenase method was used until May 2000 and the enzymatic glucose hexokinase method from that time onwards, while in Tampere a hexokinase–based method was used until September 2006 and subsequently a glucose dehydrogenase method, and in Turku the glucose dehydrogenase method was used throughout.

**4.4.4 Continuous glucose monitoring, C–peptide and self–monitored blood glucose**

All the participants in the CGM trial used the Dexcom G4 Platinum device. C–peptide concentrations were analysed by the chemiluminometric method in the Oulu University Hospital Laboratory. The glucometers used for the SMBG measurements employed the hexokinase–based method.

**4.5 Statistical analyses**

Cox regression with time–dependent covariates was used in papers I and II to evaluate the association between glucose parameters and the risk of diabetes. Univariate Cox regression analysis was used to identify risk factors for diabetes and multivariate Cox regression with the backward stepwise model was used to identify the most effective set of disease predictors.

The linear mixed model with random intercept and first–order autoregressive covariance structure for repeated measurements was used to analyse the glucose parameters over time separately for the progressors and non–progressors. In view of the long follow–up period and the division of the material between three centres, nesting of the subjects and centres was performed, and also fixing with confounding factors, as described in detail in papers I and II.

In paper III the area under the curve (AUC) for the glucose values was calculated using the trapezoidal rule. The mean amplitude of glycaemic excursion (MAGE) was calculated as previously described (391). The daily amount of carbohydrates consumed was calculated from the dietary lists kept by the families. Individual means were calculated for all the parameters tested during the follow–up week and the statistical comparisons between the age and sex–matched pairs were made with the paired sample t–test.
All the analyses were performed using IBM SPSS Statistics 20.0.0 or 22.0 for Windows, Stata/IC 11.2 or 13.1 for Windows and StatsDirect statistical software 2.7.9. The figures were drawn using OriginPro 8.6.0 and Stata/IC 11.2 or 13.1.
5 Results

We observed significant rise in the glucose parameters years before diagnosis, more specifically a rise in HbA1c 2.0 years before the clinical onset of the disease (I) and rises in OGTT and random plasma glucose 1.5 years before (II). We also characterized glucose metabolism with the CGM device in high and low–risk children and observed considerable differences in seven–day recordings. Altogether 4270 HbA1c tests, 1403 OGTTs and 3435 random plasma glucose samples were analysed during the follow–up.

5.1 HbA1c

A comparison of HbA1c levels between the groups of progressors (n=201) and non–progressors (n=265) pointed to a difference occurring 2.0 years before the diagnosis, reflecting increasing fluctuations in plasma glucose levels in the progressors, accompanied by a gradual deterioration in endogenous insulin secretion. When evaluating the potential of HbA1c for predicting the timing of disease onset three criteria were identified: a 10% rise in HbA1c values taken 3–12 months apart, an additional rise during the subsequent 6 months, and two consecutive values ≥5.9% (41 mmol/mol) in regular follow–up. The hazard ratios (HR) for these three criteria were 5.7, 5.1 and 11.9, respectively. The median times elapsing after detection of the criteria were 1.1, 0.7 and 0.9 years, respectively (I).

5.2 Oral glucose tolerance test and random plasma glucose

OGTT showed no differences in fasting plasma glucose between the two groups until very near the time of diagnosis, but the rise in 2–hour glucose values occurred 1.5 years before diagnosis. Random plasma glucose levels in the progressors started to rise 1.5 years before diagnosis whereas those in the non–progressors did not. The criteria for IFG and IGT suggested earlier by the WHO were tested and were found to be significant predictors of type 1 diabetes with high HRs of 3.2 and 8.3, and median times to diagnosis of 5.2 and 0.7 years, respectively. Rather surprisingly, random plasma glucose ≥7.8 mmol/l appears to predict type 1 diabetes almost as well as standardized OGTT, with a HR of 6.0 and a median time to diagnosis of 1.0 years (II).
5.3 Continuous glucose monitoring

The asymptomatic children with preclinical type 1 diabetes defined by positivity for \( \geq 2 \) biochemical islet autoantibodies (n=10) had higher glucose levels and higher glycaemic variation when monitored with CGM than did their autoantibody–negative controls (n=10). In particular, the time spent at or over the cut–off for impaired glucose tolerance (\( \geq 7.8 \) mmol/l) during CGM was increased in children with preclinical type 1 diabetes, accounting for 5.8% of the total time spent as compared with 0.4% for the autoantibody–negative controls. Higher variation in CGM values was observed in the high–risk children, with significantly higher standard deviation (p=0.040) and MAGE (p=0.031). Furthermore, postprandial glucose values after dinner detected by CGM and evening glucose values with SMBG were higher in these children than in the controls. The mean HbA1c was 5.7% (39 mmol/mol) in the case group and 5.3% (34 mmol/mol) in the controls (p=0.045). Plasma glucose values and C–peptide concentrations during the 5–point OGTT test showed no differences between the groups, but the sample size was relatively small (III).

5.4 Sensitivity and specificity of suggested predictive markers

The specificities and sensitivities of the suggested predictive parameters varied depending on the test used. In the HbA1c study a 10% rise in values during a 3–12 month interval showed a sensitivity of 57% and specificity 66%. When the additional rise during the next 6 months was included these became 22% and 91%, respectively, and when the results for two consecutive HbA1c values \( \geq 5.9\% \) (41 mmol/mol) were added they were 42% and 86%.

The sensitivity and specificity for IFG in the OGTT and random plasma glucose tests were 6% and 98%, respectively, those for IGT 35% and 95%, and those for random plasma glucose \( \geq 7.8 \) mmol/l 21% and 94%.

Variation in the time from seroconversion to diagnosis makes the prediction challenging, as highlighted in Fig. 4.
Fig. 4. Follow–up of HbA1c levels in 16 individuals showing a highly variable time course from seroconversion (origins of the lines) to the diagnosis of type 1 diabetes (ends of the lines).

5.5 Role of the first emerging autoantibody in the development of dysglycaemia

The distributions of IFG, IGT and random plasma glucose measurements of ≥7.8 mmol/l were analysed in the primary autoantibody groups with IAA, GADA or IA–2A as the first autoantibody. Significantly less IGTs were found in children with GADA as the primary autoantibody than in the IAA children, 9% (10/111) vs. 18% (21/117); p=0.038, and also in the random plasma glucose series the children with GADA had values of ≥7.8 mmol/l less often than did the children with IA–2A as the primary autoantibody, 8.8% (14/160) vs. 19% (7/36); p=0.047.
6 Discussion

We set out here to explore the progression of glucose intolerance in children with a high risk of developing type 1 diabetes. Our prediabetic cohort of 567 children is the largest series of young children who have participated in a long-term intensive follow-up from birth to development of diabetes-associated autoantibodies. A total of 255 of them developed type 1 diabetes during the follow-up. The large sample size allowed us to describe glucose metabolism in prediabetes accurately and reliably and also made it possible to derive predictive parameters from the glucose values.

6.1 Characterization of glucose intolerance

According to current (I, II) and previous observations (Table 2), the time period during which deteriorations in glucose metabolism can be detected varies greatly between individuals. The currently detectable rise seems to occur 2 years before the clinical diagnosis of diabetes, but this change in glucose values in an individual is undetectable until the appearance of dysglycaemia and predictive parameters such as a rise in HbA1c, a delay in the C-peptide response to an oral glucose challenge, IGT or decreased FPIR values (Table 2). These detectable changes in an individual greatly increase the likelihood of the clinical onset of type 1 diabetes (275). Given the large sample size, we were able to show that the rise in HbA1c starts 2.0 years before diagnosis (I), and those in the OGTT 2–hour value and random plasma glucose occur 1.5 years before diagnosis (II). Dysglycemia detected with parameters of 10% rise in HbA1c during 3–12 months, two consecutive HbA1c values ≥5.9% (41 mmol/mol), IGT or random plasma glucose ≥7.8 mmol/l is observed at a median time of approximately one year before diagnosis. The CGM data (III) show altered glucose values in high-risk children, with increases in variation and especially in the time spent over ≥7.8 mmol/l. We also observed both higher CGM values postprandially after dinner and higher SMBG values in the evening.

6.2 Prediction of onset

The prediction of type 1 diabetes has so far relied on the presence of islet autoantibodies, and existing metabolic markers are mainly based on OGTTs with measured C-peptide levels (392). OGTT is labour-intensive and relatively
expensive to perform and needs special preparation from the family, such as pre-test fasting and time spent at the test site. We were able here (I, II) to provide new parameters for improving the prediction of type 1 diabetes in high-risk individuals. A rise in HbA1c, dysglycaemia in OGTT and random plasma glucose ≥7.8 mmol/l are all associated with a highly increased risk of developing the clinical disease in the near future. Easy and cost-efficient repeated HbA1c measurements and a single high random plasma glucose measurement appeared to be at least comparable or better than OGTT results in our experience (II) and also in previous studies (Table 2).

The predictive parameters suggested here provide very high specificities but relatively low sensitivities. In the case of HbA1c two consecutive samples of ≥5.9% (41 mmol/mol) provided a sensitivity of 42% and a specificity of 86%, IGT 35% and 95%, and random plasma glucose ≥7.8 mmol/l 21% and 94%, respectively. When taking into account the fact that we measured HbA1c four times a year, performed an OGTT only once a year and measured random plasma glucose twice a year the figures appear more favourable for prediction purposes. All of these tests have high HRs, with a short median time to the diagnosis of type 1 diabetes. Accordingly, future follow-up studies focusing on increasing the sensitivity of the markers could start by increasing the frequency of random plasma glucose measurements, as good sensitivity could well be obtained with a relatively modest effort. HbA1c samples should be taken from high-risk individuals at follow-up visits. The only problem is that increasing the number of measurements would probably affect the specificity, and therefore efforts should be made to determine the optimal number of samples per year.

New methods such as CGM can provide still more accurate characterization of glucose metabolism and give better predictive parameters. Our high-risk individuals had readings of ≥7.8 mmol/l for 5.8% of the follow-up time, whereas for the controls these values were nearly absent with only 0.4% of the time spent at or above 7.8 mmol/l. Our CGM results also support the findings of papers I and II that HbA1c and SMBG seem to distinguish high and low-risk individuals, whereas the 5-point OGTT showed no differences between the groups. According to our findings of increased evening glucose in CGM and SMBG, it is possible that OGTTs performed in the evening could show different results than the standard tests performed in the morning after overnight fasting. Longer follow-up periods and new studies would be needed to obtain confirmation of these findings, however (III).
It has been shown previously that the first emerging autoantibody affects the time from seroconversion to clinical disease (393). We addressed the effect of the primary autoantibody on glucose metabolism in our OGTT study (II) and detected a lower frequency of dysglycaemia in children seroconverting first to GADA. Further investigations are needed with an even larger population and should be combined with histological observations if possible in order to confirm this finding regarding different subtypes.

Earlier and more accurate recognition of impending type 1 diabetes is important, since families with a child who is positive for ≥2 islet autoantibodies will obviously be concerned about the time remaining before the onset of the clinical disease. They will be well aware of the child’s high risk and will need expert counselling. Stable HbA1c measurements and OGTT or random plasma glucose results that are in the normal range will suggest that the child is not going to present with the overt disease just yet. In contrast, rises in these values will provide a warning of impending disease, which may also help to minimize the risk of severe metabolic decompensation at the time of diagnosis. The optimal interval for sampling still needs to be defined, however, in order to improve the sensitivities of these tests.

6.3 Metabolic markers in secondary prevention studies

The present results provide important information on the natural evolution of glucose metabolism in prediabetes, which can be utilized in future prevention trials. Secondary prevention trials and trials with recent–onset patients aiming at slowing down or even reversing the progression of beta cell destruction clearly need markers to monitor the disease process. The ability to predict and also stage prediabetes can help us to design smarter, shorter and less expensive clinical prevention trials by using glucose markers as inclusion criteria, given that they imply a predictable progression time to type 1 diabetes.

6.4 Shortcomings in materials and methodology

There were some potentially confounding factors in our assessments (I, II). The population was assembled by screening all newborns, but only those with an increased genetic risk were invited for regular follow–up examinations. It is not known, however, whether the HLA risk genotype itself affects glucose metabolism. Also, the children in the control group used in papers I and II were positive for ≥2
autoantibodies and the majority of them will eventually develop type 1 diabetes. As discussed above in section 2.8, “Evidence for different subtypes of type 1 diabetes”, it has been suggested that defects may occur in the size and development of the pancreas. The results presented here may therefore differ slightly if compared with a low–risk population. The non–progressor children were older at the initial seroconversion and when developing ≥2 autoantibodies, and their mean age at the end of the follow–up was higher, approximately 10 years as compared with 6.5 years in the progressor group. Also, the non–progressors less often had affected family members.

The differences between the cases and controls were mostly eliminated in paper III by using autoantibody–negative children matched for age and sex as the control group. These children also had an increased genetic risk, however, and therefore theoretically their choice affected the glucose values.

Our population (I, II) came from three clinical centres that used different methods for analysing HbA1c, OGTT and random plasma glucose. Also, the samples had been taken over a long period of time, 17 years. These differences were taken into account in the statistical analyses, as explained in the original papers. Furthermore, also a parameter denoting the relative increase in HbA1c was used in paper I to make the results more generalizable and independent of the laboratory method. In the case of OGTT and random plasma glucose (II) the laboratory method is a common one and well standardized.

The CGM trial (III) was carried out at Oulu University Hospital within one year and without any changes in laboratory methods or equipment. However, only five of the control children agreed to undergo the 5–point OGTT, and therefore the results may be misleading on account of the small population. As discussed earlier, dysglycaemia occurs at a rather late stage before diagnosis, and it is possible that the children included in the OGTT series were at an earlier stage of prediabetes, so that no differences could be observed between the groups. The follow–up of these children continues and will probably give answers later.

### 6.5 Prospects for future research

Within a few years we will be celebrating the 100th anniversary of the discovery of therapeutic insulin. Huge improvements in diabetes care have been achieved during this time (394), but despite the significant efforts the underlying causes of type 1 diabetes remain a secret. If the events triggering the onset and pathogenesis of the disease could be revealed this might help to prevent cases of type 1 diabetes or even
provide a cure. The nPOD programme offers a possibility to examine on-site events during pathogenesis, but the collection of sufficient samples will take time. Numerous trials aimed to influencing the course of the disease have been carried out and there will be more to come (Table 3).

It should be noted that several immune pathways are involved in the evolution of type 1 diabetes. This makes the designing of new therapeutic strategies aimed at controlling the immune system and preventing the destruction of beta cells a complicated matter. Some unsuccessful trials have taught us that even if one pathway to the disease is blocked, another may become more active. Thus success in prevention might require an optimal combination of currently unknown therapeutic agents. The possible targets could be components of innate immunity (such as interleukins), immunomodulatory agents targeting adaptive immunity (anti–CD3, anti–CD20), possibly the induction of regulatory T cells and tolerogenic dendritic cells, or the increasing of beta cell mass and its regeneration (GLP–1). It may also be necessary to take the individual disease process into account in order to achieve success, as discussed in chapter 2.9, “Prevention of type 1 diabetes”. And of course, careful design with an adequate sample size will be essential.

The staging of type 1 diabetes will become important in order to achieve sufficient sample sizes and to follow the responses to treatment properly. The ability to recognize high-risk individuals with metabolic markers can provide a framework for research and the development of preventive therapies. In fact the staging of prediabetes and predictive glucose parameters can already be used as inclusion criteria for clinical trials in order to achieve smaller sample sizes and shorter follow-up times, which is particularly important in the absence of effective interventions and when the available combinations of therapeutic agents are more or less experimental. The search for novel and more accurate biomarkers, along with glucose parameters, is continuing in the hope of being able to predict the onset of the disease and recognize the stages of individuals presenting with prediabetes.
7 Summary and conclusions

We confirmed here that a deterioration in glucose metabolism occurs approximately two years before the clinical onset of type 1 diabetes. We also found that the differences in glucose metabolism between autoantibody positive and negative individuals can be highlighted with CGM, and that the prediction of type 1 diabetes can be improved by means of at least the following glucose parameters.

1. HbA1c values start to rise 2.0 years before the clinical onset in autoantibody–positive individuals, so that the onset can be predicted with 10% rise, or two consecutive samples of HbA1c \(\geq 5.9\% \text{ (41 mmol/mol)}\).
2. OGTT values at 2 hours rise 1.5 years before diagnosis and the onset can be predicted with detection of IGT in risk children.
3. Random plasma glucose rise 1.5 years before the diagnosis of type 1 diabetes in risk children, and the onset can be predicted with detection of plasma glucose \(\geq 7.8 \text{ mmol/l} \).
4. Children with \(\geq 2\) biochemical autoantibodies have higher glucose values and increased variation in CGM and SMBG measurements.
5. Increased evening glucose values in CGM and SMBG seem to be common in children with preclinical type 1 diabetes.
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Original publications


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1368. Krökki, Olga (2016) Multiple sclerosis in Northern Finland: epidemiological characteristics and comorbidities
1369. Mosorin, Matti-Aleksi (2016) Prognostic impact of preoperative and postoperative critical conditions on the outcome of coronary artery bypass surgery
1372. Helikala, Anne (2016) Ketoacidosis at diagnosis of type 1 diabetes in children under 15 years of age
1375. Lehtonen, Ville (2016) Dental and otologic problems in cleft lip and palate patients from Northern Finland: cleft associated problems
1376. Koivukangas, Jenni (2016) Brain white matter structure, body mass index and physical activity in individuals at risk for psychosis: The Northern Finland Birth Cohort 1986 Study
1377. Väyrynen, Sara (2016) Histological and molecular features of serrated colorectal adenocarcinoma and its precursor lesions
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GLUCOSE METABOLISM IN PRECLINICAL TYPE 1 DIABETES